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STUDIES OF EPIGENETIC INSTABILITY IN HUMAN NORMAL AND DISEASED VULVA SKIN

A Thesis
Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements of the degree of
Master of Science

In

The Department of Biological Sciences

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August, 2006

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ABSTRACT

Epigenetic modification is another mechanism involved in the cancer development besides classic mutations such as deletion. Aberrant promoter methylation and associated chromatin modification have been frequently reported in various tumors of different clinical stages. Hypermethylation has been frequently observed in tumor suppressor genes and causes reduced transcripts of these genes. Hypomethylation also has been reported to be involved in activation of oncogenes. Inactivation of the X chromosome and the imprinting of gamete DNA depend on the methylation patterns in the promoter region. Understanding methylation mechanisms could be helpful to diagnosis of early stage tumorgenesis or offer molecular markers for detecting cancers.

Changes in methylation patterns based on human vulva pathological tissue type of four genes have been studied in the current project. RASSF1A and DAPK-1 are tumor suppressor genes. BRCA2 is considered highly associated with breast and ovarian cancer. And H19 is a maternal imprinted gene. DAPK-1 and BRCA2 have been found to be significantly hypermethylated in Lichen Sclerosis (LS) and Squamous Cell Carcinoma (SCC). And RASSF1A has displayed an interesting methylation pattern in the post transcription region, where the frequency of methylation significantly decreased from normal tissue to LS tissue, but then dramatically increased to SCC. H19 failed to show any changes in the methylation pattern with methods tested. This was most likely due to interference of primer dimers by SYBR-green in the real-time PCR analyses.

Instead of using the same promoter sequence reported by previous papers, extended estimated promoter regions and partial transcription regions were obtained from Genome

Browser and additional CpG island sites have been studied in current project. Methylation patterns have been detected that differ from the literature in these genes. These results may provide more information to find a more precise active promoter region and epigenetic involved sequences for future research.

CHAPTER ONE
INTRODUCTION

1.1. Epigenetic Modification and DNA Methylation

The genetic information of an organism is written as a four-character code in DNA, and is highly regulated and organized. However, epigenetic modification may change the phenotype of a cell without altering its genotype, and that type of modification may also be heritable. DNA methylation associated with histone modification is the only known type of epigenetic modification essential in controlling gene expression under normal circumstances in human and mammalian cells (Biel et al. 2005).

DNA methylation in eukaryotes always occurs at the 5 position of cytosine residues followed by a guanosine residue in a CpG dinucleotide configuration, but in some bacteria cells, methylation also takes place at adenine residues (Singal and Ginder 1999). Once cytosine residues have been methylated, accidental deamination leads to the conversion of methylcytosines to thymine residues which challenge DNA repair mechanisms (Antequera and Bird 1993). Therefore, the frequency of CpG normally is pretty low throughout the entire genome, representing only one base in around 1 per 50-100 (Feltus et al. 2003). Approximately 70% to 80% of the CpG sites contain methylated cytosines in most vertebrates, including humans (Bird 1995).

CpG islands are regions that are unusually rich in CpG sequences which range from 0.5 to 5 kb in length and occur on average every 100 kb with distinctive properties (Bird 1992). These regions are mostly unmethylated, GC rich (60% to 70%), and have a ratio of CpG to GpC of at least 0.6 (Cross and Bird 1995). There are at least 29,000 such regions in

the human genome, many of which reside in the 5'end of genes (Feltus et al. 2003). Chromatin contained in CpG islands is generally heavily acetylated, lacks histone H1, includes a nucleosome-free region which may allow or be a consequence of, the interaction of transcription factors with gene promoters (Singal and Ginder 1999). Each gene builds its own methylation pattern through specific methyltranferases and maintains its pattern through mitosis. Aberrant methylation in the promoter regions of genes interferes with gene expression and may be involved in many human diseases. Figure1.1 shows the biochemical pathways for cytosine methylation, demethylation, mutagenesis of cytosine and 5-mC and the enzymes involved in those pathways.

1.2. Functions of DNA Methylation

1.2.1 Roles in Gene Expression

1.2.1.1 Regulating Gene Expression

Experiments have shown that DNA methylation in the promoter region is related to gene transcriptional repression and gene silencing. Although the real mechanism of how DNA methylation results in gene silencing is still unclear, three possibilities have been proposed. First, DNA methylation might directly interfere with the binding of specific transcription factors to their recognition sites in their respective promoters (Tate and Bird 1993).Second, it might cause the direct binding of specific transcriptional repressors to methylated DNA(Rountree and Selker 1997). Lastly, it also might alter chromatin structure such as deacetylation of the core histones H3 and H4 (Singal et al. 1997). However, whether methylation is a primary control mechanism or a secondary effect, gene silencing is still an important issue in human disease that needs to be addressed (Singal and Ginder 1999).

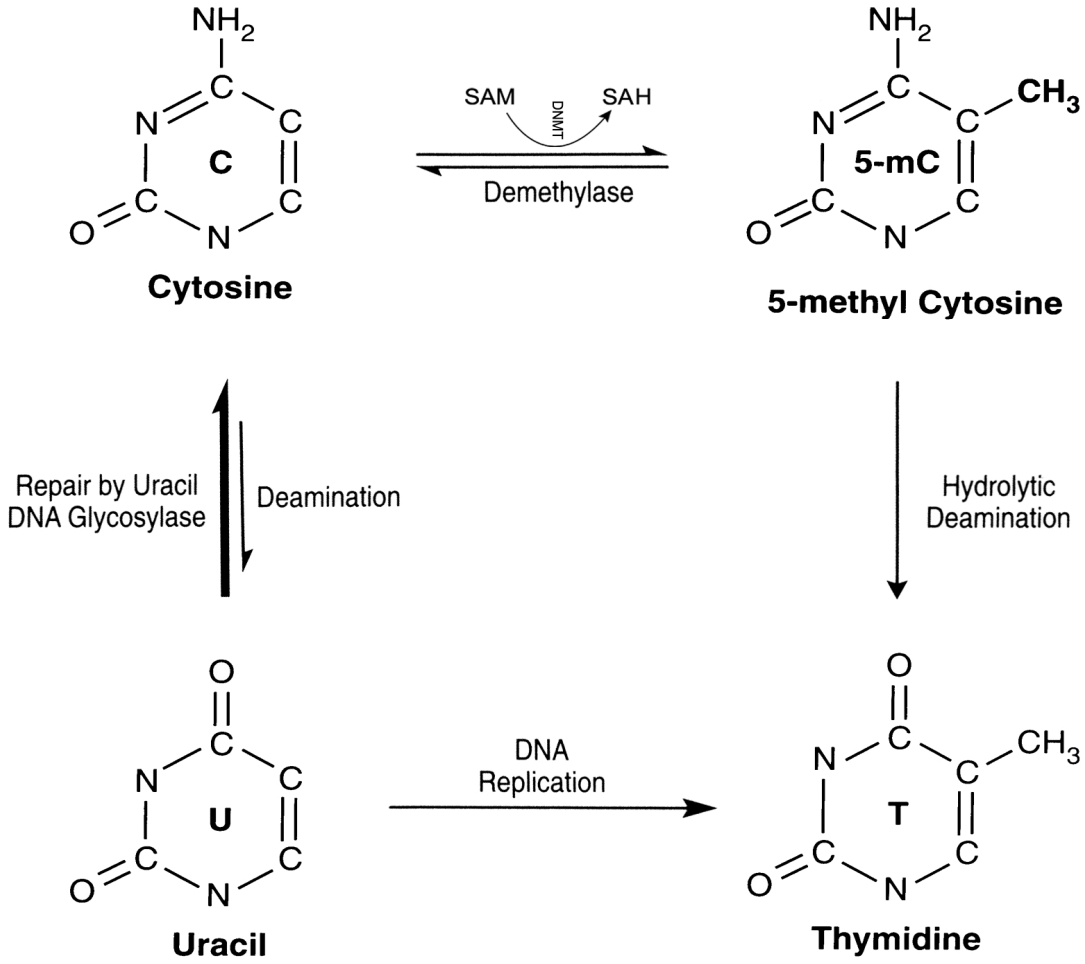

Figure1.1. Schematic representation of the biochemical pathways for cytosine methylation, demethylation, and mutagenesis of cytosine and 5-mC (modified figure from Singal and Ginder 1999).

1.2.1.2 Involved in Cancer (Carcinogenesis)

The patterns of DNA methylation are gene-specific, and tissue specific, so that a unique set of proteins are only expressed to perform functions specific for a certain type of cell. Therefore, DNA methylation patterns play a critical role in silencing specific genes during development and cell differentiation (Singal and Ginder 1999). Numerous studies have shown that aberrant methylation patterns in the promoter regions are among the earliest and most common events in tumorigenesis. Activation of proto-oncogenes through hypomethylation, transcriptional inactivation of tumor-suppressor genes through

hypermethylation, and defects in chromosomal segregation due to failure of de novo methylation may all contribute to neoplasia (Singal and Ginder 1999).

Hypomethylation may be an important etiological component of cancer development. Experiments have shown that hypomethylation is involved in gene activation, repetitive elements de-repression, and chromosomal instability (Scarano et al. 2005). Global hypomethylaiton was the first epigenetic abnormality to be identified in cancer cells (Scarano et al. 2005). Experiments have also shown that promoter hypomethylation is associated with cancer by activation of expression of normally silenced genes such as oncogenes (Chen et al. 1998). H-ras which regulates signal transduction is an important example of a hypomethylated oncogene (Feinberg and Tycko 2004).

Hypermethylation is opposite to hypomethylation which refers to the increasing level of methylation. Since aberrant promoter hypermethylation could inactivate normally expressed genes, it contributes to silencing of tumor suppressor genes (Singal and Ginder 1999). Aberrant promoter hypermethylation has been found to be involved in almost all the important steps of carcinogenesis: cell-cycle regulation, DNA repair, drug resistance and detoxification, apoptosis, cell differentiation, angiogenesis and metastasis (Scarano et al. 2005). A lot of tumor suppressor genes such as retinoblastoma gene (Rb), p16, P53, VHL, MLH1, and BRCA1 have been detected to be involved in transcriptional inactivation in cancer cells (Lim et al. 2003). The retinoblastoma gene (Rb) was the first classic tumor-suppressor gene to be detected with promoter hypermethylation (Lim et al. 2003). P16 is known as one of the most important cell cycle regulatory proteins, and hypermethylation mediated inactivation of the p16 gene has been demonstrated in brain, breast, colon, head and

neck, and non-small-cell lung cancer and in high grade non-Hodgkin's lymphoma (Baylin et al. 1998). Mutations in the p53 tumor-suppressor gene occur in more than 50% of human solid tumors (Greenblatt et al. 1994).

1.2.2 Genomic Imprinting and X Chromosome Inactivation

Genomic imprinting is a particularly important mechanism in mammals. Genomic imprinting refers to the expression of a gene in the developing embryo being dependent on the allele's parental origin (Feinberg et al. 2002). Over 70 imprinted genes have been identified in mammals so far (Murphy and Randy 2003). DNA methylation has been found to be the most consistent difference between the alleles of an imprinted gene, accompanied by differences in chromatin conformation, histone modification, replication timing and recombination rate (Paulsen and Ferguson-Smith 2001).

The reason for imprinting has not been conclusively identified todate, but the most prominent assumption is that this process is necessary for development and may somehow regulate growth in the embryo and behavior after birth (Paoloni-Giacobino and Chaillet 2004). Another theory based on evolution proposes that imprinting reflects the competing interests of the maternal and paternal genomes in the developing embryo (Murphy and Randy 2003).

In mammals, the random inactivation of one X chromosome also occurs by methylation and histone H4 deacetylation (Scarano et al. 2005). This pattern is initiated at blastocyst stage, around the time of implantation, and is maintained in all somatic cells throughout (Maxfield Boumil and Lee 2001).

Loss of imprinting (LOI) is the disruption of imprinted epigenetic marks through gain

or loss of DNA methylation (Kaplan et al. 2003). Studies of LOI have shown it plays roles in cancer. LOI of IGF2 is the most common event across the widest range of tumor types including colon, liver, lung, and ovarian cancer, as well as Wilms' tumor which is the embryonic kidney cancer where LOI was first discovered (Robertson 2005). Research has shown that imprinting plays a role in the development of disease. (Peterson and Sapienza 1993) reviewed various disease syndromes caused by aberrant imprinting. The best characterized syndromes related to growth and behavioral defects are Beckwith–Wiedemann syndrome (BWS) on chromosome 11p and the Prader–Willi/Angelman syndrome (AS) on chromosome 15q.

1.2.3. Roles in Evolution

From prokaryote, to nonvertebrate eukaryotes and vertebrates, the size of the genome has increased from a few thousand genes to greater than 30,000genes (Bird 1995). And the frequency of methylation of cytosine occurs much less in invertebrates to none in some species such as *Drosophila* (Singal and Ginder 1999). (Bird 1995) proposed that since the greater the number of tissue specific genes, the greater the efficiency of gene transcription required. Therefore, DNA methylation in vertebrates reduces the transcriptional noise through a global repression, and thereby allows vertebrates to accumulate and selectively use the extra genes that are crucial to their development. DNA methylation is also considered as a type of cell self-defense system during evolution(Yoder et al. 1997). It can inactivate the promoter of most viruses and transposons, including retroviruses and Alu elements (Singal and Ginder 1999). In addition, specific endonucleases may recognize foreign DNAs since they have a different methylation pattern from host cells, and thus

destroy those exogenous DNA fragments (Yoder et al. 1997).

1.3. Enzymes Involved in DNA Methylation

The methyl group is transferred from S-adenosylmethionines (SAM) to the 5 carbon of cytosine ring through the action of DNA methyltransferses (DNMT), and SAM is converted to S-adenosyl-homocysteine (SAH) (Campbell and Szyf 2003). Figure 1.2 illustrates the mechanism of DNMT methylation via the SAM pathway (Strathdee and Brown 2002). DNA methyltransferases establish the methylation pattern through de novo methylation, and then maintain the specific pattern after semi-conservative DNA synthesis (Bestor 2000).

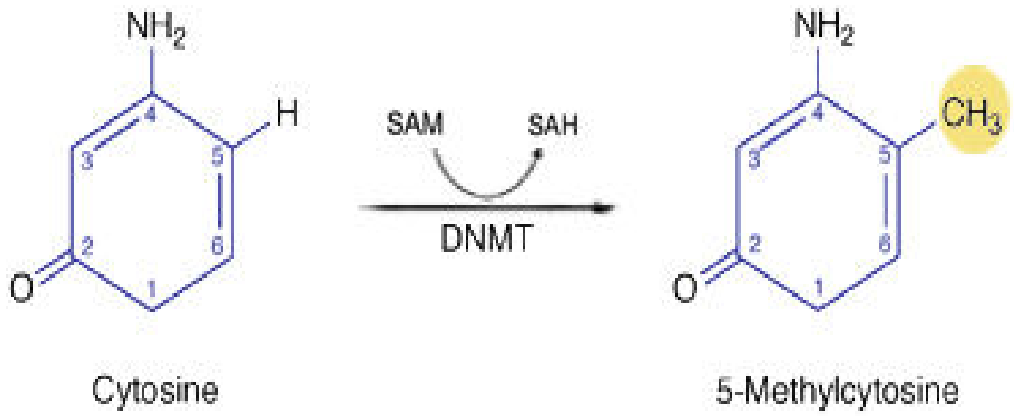

Figure1.2. Mechanism of DNA methylation.5-Methylcytosine is produced by the action of the DNA methyltransferases (DNMT 1, 3a or 3b), which catalyze the transfer of a methyl group (CH3) from S-adenosylmethionine (SAM) to the carbon-5 position of cytosine. (modified from Strathdee and Brown 2002) .

Three methyltransferases genes (*DNMT1, DNMT3A, DNMT3B*) and one candidate gene, *DNMT2*, have been identified. *DNMT1,* the first one identified and cloned, is highly conservative in eukaryotes, and responsible for maintaining DNA methylation patterns during DNA replication (Figure1.3) (Robert et al. 2003). It plays essential roles in X-inactivation, genomic imprinting and genome stabilization (Bestor 2000). DNMT3A and

DNMT3B are essential for de novo methylation and play important roles in normal embryonic development and disease such as ICF syndrome (Okano et al. 1999). DNMT2 has been identified in human and mouse which appears to lack the large N-terminal regulatory domain common to other eukaryotic methyltransferases (Yoder and Bestor 1998). Recent studies also have suggested aberrations in DNA methyltransferase activity in tumor cells (Singal and Ginder 1999).

Besides methyltransferases, another enzyme called demethylase has been proposed to direct the removal of the methyl group from methylated CpG (Bestor, 2000). However, no demethylase enzyme or gene has been identified or isolated to date.

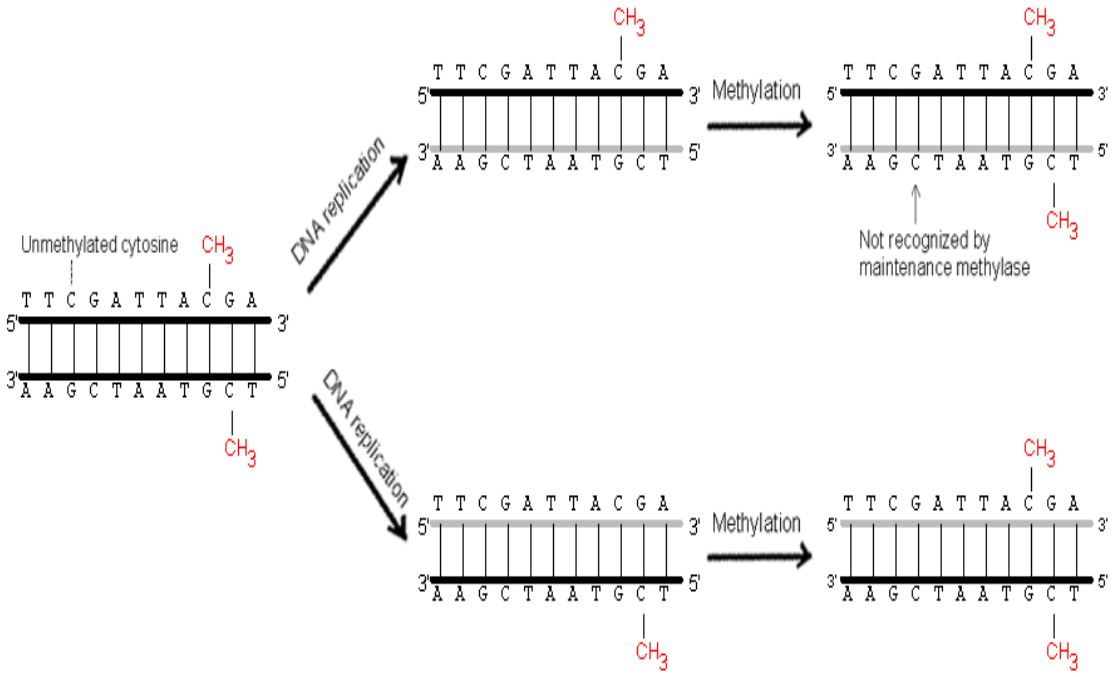

Figure1.3: Inheritance of the DNA methylation pattern.The original methylation pattern is maintained through mitosis The maintenance methylase can methylate only the CG sequence paired with methylated CG. The CG sequence not paired with methylated CG will not be methylated.

(modified from www.web-books.com/MiBio/Free/ch7F2.htm)

1.4. Causes of Aberrant Methylation

Disruption of normal DNA methylation patterns may occur as a consequence of exposure to toxic agents, disease processes, and/or nutritional deficiencies.

Along with dietary factors, aging, chronic inflammation, viral infection, and exposure to epimutagens all may cause aberrant methylation (Ushijima and Okochi-Takada 2005). Among those, aging, chronic inflammation, viral infections, chemicals including nickel, butyrate, and arsenic can induce aberrant methylation. Folate deficiency, choline deficiency, and 5-aza-2'-deoxycytidine (5-zaz-dC) and its derivatives cause hypomethylation(Ushijima and Okochi-Takada 2005).

Folate is a water soluble B vitamin and an important mediator in methyl group metabolism (Kim 1999). 5-Methyl-terahydrofolate is a precursor of SAM and is the primary methyl donor for most biological methylations (Sohn et al. 2003). A lack of folate could increase the risk of cancer (Trasler et al. 2003).

1.5. Studies of Epigenetic Instability

Since DNA methylation is involved in gene regulation, evolution, cancer development, genetic imprinting and X chromosome inactivation, and it is also unlike mutational inactivation. Methylation is reversible and demethylating agents and inhibitors of histone deacetylases could be used in clinical trails, knowing the mechanism of DNA methylation may therefore have important clinical implications for the prevention and treatment of human diseases. Studies of DNA methylation have been performed for several decades, but the understanding of the whole puzzle is still very limited. The relationship between gene regulation, DNA methylation, histone deacetylation, histone acetylation, methyltransferases,

demethylases, methyl cytosine binding proteins, and the transcriptional activity of genes are the necessary information to understanding epigenetic mechanisms and need to be fully studied.

Epigenetic instability is measured by the detection of abnormal methylation of DNA sequences, primarily promoter regions of oncogenes, tumor supressor and imprinting genes. These procedures are based on PCR and MS-PCR (methylation specific-PCR) techniques and gel electrophoresis, and occassionally include restriction endonuclease digestion. DNA is treated with bisulfite to convert non-methylated cytidine residues to uridine (and then thymidine upon PCR amplification), while the methylated cytidine (5-methyl-cytidine) residues remain unchanged(Frommer et al. 1992). These procedures are well published and the techniques are currently in use in our laboratory.

The present project will enable the study of the methylation status of genes from normal skin to a preneoplastic disease (lichen sclerosus) state to squamous cell carcinoma. I begun this work with PCR primer sets specific for methylated and unmethylated sequences that are reported in the literature for each gene. New primers were designed where necessary for the development of a more sensitive DNA methylation assay. Methylation Specific PCR is used to distinguish methylated from unmethylated DNA in this assay.

1.6. Gene Selection

Four genes were chosen for methylation analyses: DAPK- 1, RASSF1A, BRCA2 and H19. Numerous publications in the literature have demonstrated the common occurrence of hypermethylation of tumor suppressor genes in diagnosed cancers(Looijenga et al. 1997;

Dammann et al. 2000; Reddy et al. 2003; Robertson 2005). But no one has verified the status of those methylation changes early in the tumorigenic process of skin squamous cell carcinoma (SCC). RASSF1A and DAPK-1 have been reported to be hypermethylated in cancers, and are studied here to for their methylation status in skin cancers. Although H19 is an imprinted gene, no one has reported it being hypomethylated in human cancer. BRCA2 has been reported to be involved in breast cancer, but the methylation status in skin cancers has not been reported yet.

1.6.1 RASSF1A

The RASSF1 gene locus spans about 11,151 bp of the human genome which includes eight exons, and differential promoter usage and alternative splicing generated seven isomers (A-G) (Agathanggelou et al. 2005). RASSF1A is located at the 3p21.3 locus and is considered to be a tumor suppressor gene which is frequently inactivated by epigenetic events rather than classic mutation/deletion events (Vos et al. 2004; Agathanggelou et al. 2005). RASSF1A has been reported to be involved in apoptotic signaling, microtubule stabilization and mitotic progression (Dammann et al. 2005). However, the precise mechanisim of how RASSF1A protein functions in cell cycle regulation and tumor suppression remains unknown (Vos et al. 2004).

Abberant hypermethylation of the RASSF1A promoter region has been reported in a number of human primary tumors (>90 PubMed entries) and was observed initially in lung cancer (Dammann et al. 2000). Those tumors include epithelial origin tumors such as breast, cervix, liver, small cell lung carcinomas, nasopharyngeal carcinomas, renal cell carcinomas, prostate carcinomas, and non-epithelial origin tumors such as neuroblastomas (Spugnardi et

al. 2003). Hypermethylation of the promoter region also has been detected in body fluids including blood, urine, nipple aspirates, sputum and bronchial alveolar lavages (Dammann et al. 2005). Therefore, detection of aberrant RASSF1A methylation may serve as an early diagnostic and prognostic marker for cancer.

1.6.2 DAPK1

Death-associated protein kinase 1 (DAPK1) is located in 9q34.1, and it is a 160-kDa calcium/calmodulin-dependent serine-threonine kinase. It is required for γ interferon-induced apoptosis and is considered to be a tumor suppressor gene (Lehmann et al. 2002). Previous studies have shown that treatment with 5-aza-2'deoxycytidine (a demethylation reagent) on non-expressing cell lines resulted in expression of DAPK-1 gene product, which suggested epigenetic invovlment (Katzenellenbogen et al. 1999). Numerous studies have shown silencing of DAPK due to promoter hypermethylation plays role in lung, esophageal, head and neck, prostate, bladder, gastric, and B-cell malignancies including B-cell lymphoma, multiple myeloma, and Burkitt's lymphoma (Reddy et al. 2003). However, the timing of DAP-kinase inactivation by methylation during tumor development has not been established (Pulling et al. 2004).

1.6.3 H19

H19 is the first human gene recognized to be paternally imprinted where the paternal allele is inactivated through methylation (Brannan et al. 1990). It is located in chromosome 11p15.2 in both mice and humans within a cluster of at least five imprinted genes, including IGF2 (Zemel et al. 1992; Morison et al. 2001). IGF2 and H19 are reciprocally imprinted and coordinately regulated by an intergenic imprinting center and a common

enhancer region (Kopf et al. 1998). IGF2 is an autocrine growth factor with an important role in many types of cancer, and H19 is a non-coding RNA of unknown function with growth suppressive properties, possibly functions as a tumor suppressor or oncofetal gene (Hao et al. 1993). Loss of imprinting (LOI) of H19 has been observed in human malignancies, including lung cancer (Kaplan et al. 2003). But it is also interesting that up-regulation of H19 without LOI was found in the airway epithelium of cigarette smokers (Kaplan et al. 2003). H19 is normally expressed during embryogenesis at high levels in many organs (Lustig et al. 1994). In humans, there is biallelic expression of H19 in the placenta at <10 weeks of gestation, but expression becomes monoallelic after 18-20 weeks (Zhang and Tycko 1992). In adults, H19 expression remains monoallelic, with expression primarily in skeletal muscle, thymus, heart, and lung (Looijenga et al. 1997).

1.6.4 BRCA2

BRCA1 and BRCA2 are two breast cancer susceptibility genes which have become integrated into the practice of clinical oncology (Narod and Foulkes 2004). BRCA1 is located at chromosome17q21, and BRCA2 is located at 13q12.3 (King MC 2001).

BRCA1 plays roles in DNA repair, cell-cycle-checkpoint control, ptotein ubiquitylation and chromatin remodeling (Scully and Livingston 2000). However, BRCA2 is only known to be involved in homologous recombination (Narod and Foulkes 2004). Hypermethylation of the BRCA1 promoter has previously been shown to cause reduced mRNA expression in both breast and ovarian cancer (Chan et al. 2002), but hypomethylation of the BRCA2 promoter region has been demonstrated in sporadic ovarian cancer (Chan et al. 2002). No paper has report hypermethylaiton of BRCA2

associated with reduced mRNA expression yet. Studies have found high frequencies of hypermethylation of BRCA1 and hypomethylation of BRCA2 occurred within the same specimens, so both of them may be important in the development of sporadic ovarian cancer (Chan et al. 2002).

1.7. Pathological Types of Skin

1.7.1 Lichen Sclerosus (LS)

Lichen sclerosus (LS) is a chronic inflammatory dermatosis that is not contagious (Boms et al. 2004). Any skin may be involved but the most common site is the genitalia which represents around 85% to 98% of cases (Oyama et al. 2004). It has been reported that LS affects women 6 to 10 times more often than men (Arican et al. 2004). The affected skin is often itchy, usually white and sometimes with fine, crinkling texture (Arican et al. 2004). As the condition progresses, bruising and pain may occur (Arican et al. 2004). Although it is still not fully understood what causes lichen sclerosus (LS), it is generally thought that it is not caused by a virus, bacteria, or fungus but something else entirely. Patients who with untreated disease have an increased risk of a skin cancer of the involved areas, which amounts to around 5% of cases that progress to malignancy most frequently squamous cell carcinoma (Oyama et al. 2004).

1.7.2. Squamous Cell Carcinoma

American Academy Dermatology (http://www.aad.org/) states that squamous cell carcinoma (SCC), the second most common skin cancer after basal cell carcinoma, afflicts more than 250,000 Americans each year. It arises from the epidermis and resembles the squamous cells that comprise most of the upper layers of skin and may occur on all areas of

the body including the mucous membranes. SCC is mainly caused by UV light (http://www.skincancer.org/). Squamous cell carcinomas usually appear as crusted or scaly patches on the skin with a red, inflamed base, a growing tumor, or a non-healing ulcer (http://www.aad.org/). SCCs are usually locally destructive but if it left untreated, it eventually penetrates and invades the underlying tissues. In a small percentage of cases, SCCs spread (metastasize) to distant tissues and organs. When this happens, SCCs can be fatal (http://www.skincancer.org/).

CHAPTER TWO
MATERIAL AND METHODS

2.1 Sample Collection and Labeling

Human vulva tissues from over 100 patients were provided by Dr. Andrew Carlson of Albany Medical College. Squamous carcinoma with adjacent lichen sclerosis and surrounding normal tissues were obtained from each patient following therapy prescribed radical vulvectomy; all of the surgically resected tissues were histologically analyzed. Normal tissues from patients with no apparent vulva ailment were also collected and used as uninvolved sample controls. Specimens were collected under approved IRB protocols with informed consent from all surgical patients. Tissues were separately labeled and packed based on the histological types and patients. Then tissues specimens were shipped on dry ice and stored at -80 °C until further analysis.

Letter "CW" were assigned to samples from individuals with histological types associated with cancer (SCC), lichen sclerosis (LS), and normal adjacent tissues. The number after "CW" represents the patient's number. A different number refers to different individual. The sub-grouping (last number in the sequence) is an identifier used to discriminate multiple tissues taken from one patient.

2.2 Genomic DNA Preparation

Two different DNA extraction procedures were used to purify DNA from samples. The first one was a standard phenol and choloroform extraction protocol which is a universal technique and the details are listed in appendix A. Equal volume phenol and choloroform are used to remove proteins (e.g., Dnases and other enzymes) and followed by precipitation of

the DNA. Details of this protocol have been listed in appendix A. The second procedure utilized the QIAamp DNA Mini Kit (Qiagen Inc., Chatsworth, CA) which makes the extraction of DNA more efficient. In both protocols, DNA are precipitated by cold ethanol and re-suspended in 1x TE buffer (Appendix B). The isolated DNAs were quantified through a Beckman DU-64 Spectrophotometer.

2.3 Bisulfite Treatment of Isolated DNA

The purpose of this published procedure (Frommer et al. 1992) is to convert unmethylated Cytosine to Uracil, without changing the methylated cytosine residues (Figure 2.1). After the modification, the two daughter strands of modified DNA will no longer be complementary to each other. For the present study, approximately 1-2µg of DNA was denatured in NaOH and modified by sodium bisulfite treatment following the protocol of (Herman et al. 1996). Then the modified DNA samples were purified by using the Wizard DNA purification kit (Promega), treated again by NaOH and precipitated by cold ethanol. Dry DNA pellets were stored at -80°C and re-suspended in 20µl of 0.1X TE. Details of this protocol have been listed in appendix C.

2.4 Methylation Specific PCR

2.4.1 Gene Specific Sequences

Gene promoter sequences were originally obtained from published papers identified on PubMed, but after comparison by BLAT (http://www.genome.ucsc.edu/cgi-bin/hgBlat), the published sequences were found to be incomplete with modified mRNA sequences. Therefore, Genome Browser(http://www.genome.ucsc.edu) was used to search the complete gene sequences. Promoter sequences were estimated to be 1000bp upstream from the first

exon of each gene.

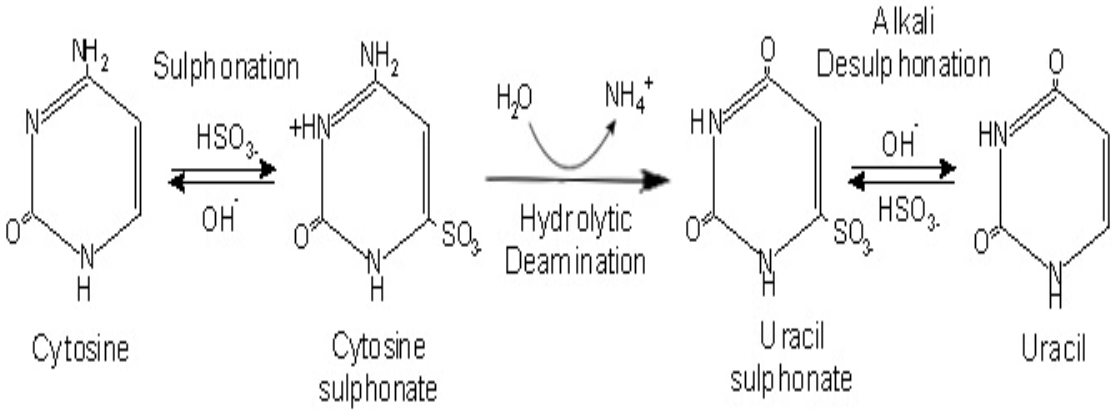

Figure 2.1 The Mechanism of Bisulfite Treatment.
(modified from **www.methods-online.net**)

2.4.2 Methylation-Specific PCR Primer Design

Methprimer (http://www.ucsf.edu/urogene/methprimer/) was used to find out the regions of CpG islands within estimated promoter sequences and partial cDNA sequence. MethPrimer, which is based on Primer3, is a program designed for the production of MSP primers (Li and Dahiya 2002).

Primers for RASSF1A and DAPK were originally chosen based on published papers (Herman et al. 1996; Lehmann et al. 2002), but PCR amplifications lacked the required specificity. Therefore, Methprimer was used to design primer sets for both methylated and unmethylated sequences for all the four genes. Primers were designed within each CpG island and incorporated at least 3 non-CpG cytosines in the original sequence to assure that unmodified DNA will not serve as a template for the primers. The last three bases at the 3'-end of primers contained at least one CpG sequences. This provides optimal specificity and minimizes false positives due to mispriming. Primers in the M (methylated) pair and U

(unmethylated) pair also contained the same CpG sites within their sequence. Both M and U sets of primers should preferably have similar *Tm* values, thus allowing the two PCR reactions for each sample to be carried out simultaneously in the same PCR reaction under the same annealing conditions.

2.4.3 Methylation Specific PCR Amplification

After bisulfite conversion, the modified DNA was ready to be used as a template for U and M PCR analyses. Methylation PCR (M primer set) was designed to be specific for DNA originally methylated for the gene of interest, and unmethylation PCR (U primer set) was designed to be specific for DNA originally unmethylated. PCR products were separated on 2% agarose gels and the bands were visualized by staining with ethidium bromide using UV fluorescence. The presence of a band indicates the presence of unmethylated, and/or methylated alleles, in the original sample. MSP was very sensitive, and permitted the analysis of small and heterogeneous samples, including paraffin-embedded material.

Twenty µl PCR amplifications were performed under the following conditions: 2µl of modified DNA (50-100ng), 1µl of primers (final concentration is 0.125µM), 10µl of TAKARA Real Time Pre-Mix (Takara Mirus Bio) and 7µl of water. All reactions were subjected to the an initial denaturation step at 95ºC for 30 seconds. If the annealing temperature >=60ºC, then a shuttle PCR was performed where each cycle has a 5 seconds denaturation step at 95ºC followed by a 34 seconds primer annealing step at the specified annealing temperature. If the annealing temperature is <60ºC, then after 5 seconds denaturation step at 95ºC, a 20 seconds annealing step at the specified annealing temperature is performed followed by a 30 seconds extension step at 72ºC.

2.4.4 Real-time Methylation Specific PCR

The real-time PCR system is based on the detection and quantitation of the signal of a fluorescent reporter which increases in direct proportion to the amount of PCR product in a reaction at each cycle (Lee et al. 1993; Livak et al. 1995). The higher the starting copy number of the nucleic acid target, the sooner a significant increase in fluorescence is observed. The cycle number at which the fluorescence emission exceeds the fixed threshold is defined as C_T. Therefore, the more initial target templates present, the smaller the C_T value is. The difference between the C_T of each sample indicates the difference between the initial sample size or initial copy number.

Since partial hypermethylation or partial hypomethylation could not be detected by traditional MS-PCR, real-time MS-PCR turns out to be the appropriate way to detect the difference between initial amount of unmethylated amplicons and methylated amplicons quantitively.

SYBR green was used as a reporter dye in the real-time PCR. It is a fluorogenic minor groove binding dye that exhibits little fluorescence when in solution but emits a strong fluorescent signal upon binding to double-stranded DNA (Morrison et al. 1998). The disadvantage of this reporter dye is that none-specific PCR products interfere with the target signal. But SYBR green is a less expensive reagent which is ideal if there is no non-specific PCR product.

Real-time PCR amplifications were performed by using ABI PRISM® 7000 Real- PCR system. 2µl of modified DNA (50-100ng), 0.6µl of primers (final concentration is 75nM), 10µl of ABSOLUTE™ QPCR SYBR® Green Mixes (ABgene Inc., USA) and 7.4 µl of

water end up 20 µl as total per reaction. All reactions were subjected to the an initial denaturation step at 95ºC for 15 minutes. The parameters for the rest of the cycles are same as those described in Methylaiton Specific PCR.

A dissociation curve analyses starting at 60ºC has to be performed after real-time PCR amplification steps. Dissociation curve is used to detect the presence of non-specific amplification products in SYBR-green real-time PCR.

Three replicates of each DNA sample were analyzed at the same time. Unmethylation and methylation real-time PCR were also carried on the same plate at the same time. The average value of C_T from each sample was calculated and compared. Table 2.1 lists primer names, sequences, product base pair size, annealing temperatures, and sources.

2.5 Reagents

The TAKRA polymerase (Premix Ex Taq™) was purchased from Takara Mirus Bio which is a newly-formed joint venture between Takara Bio Inc. and Mirus® Corporation. ABSOLUTE™ QPCR SYBR® Green Mixes were purchased from ABgene Inc., USA. The primers used in the assay were purchased from Integrated DNA Technologies, INC (IDT). Primers were diluted upon arrival with 1X TE buffer to 100µM as stock. Primer stocks were further diluted to 2.5 µM before use in PCR anaylses. Unmethylated and methylated controls were purchased from Serologicals Corporation (United Kingdom). All reagents were stored in a standard freezer at -20°C and thawed on ice before use.

2.6 Statistics Analysis

For MSP, Chi-Square Test was used to determine if the methylation patterns were significantly different between the tissue types. For real time PCR, one way ANOVA was

performed, and tissue type was considered as fixed effects. This statistical analysis determined if different pathological types had significant differences regarding to the methylation pattern. A p value which is equal to or less than 0.05 is considered to demonstrate a statistically significant difference between pathological types.

Table 2.1 Primer sets of all the genes

Primer Set	Forward Primer 5'→ 3'	Reverse Primer 5'→ 3'	Size bp	Annealing Temp(^{o}C)
(1,2)RASSF1A-U	TAAATTGGATTAGG AGGGTTAGGGTT	CCCAAAATCCAAACT AAACAACAAA	290	61
(1,2)RASSF1A-M	ATCGGATTAGGAG GGTTAGGGTC	CCAAAATCCAAACTA AACGACGA	289	61
(2)RASSF1A-U	GGTAGTTGGTTTTT GGTTGTGGTTATT	ACCTAATCCTCAAAA ACTATCCCCAC	237	61
(2)RASSF1A-M	TGGTTTTTGGTCGT GGTTATCGT	TCCTCGAAAACTATCC CCGC	225	61
(1)DAPK1-U	AGTATTTTGGGAGG TTTAGGTGG	TCCCCAATAACTAAA ACTACAATCAC	145	56
(1)DAPK1-M	TTAGTATTTTGGGA GGTTTAGGC	TCCCCAATAACTAAA ACTACAATCG	147	56
(2)DAPK1-U	AGTTGTGTTTTTGTT GTTGTTTTGG	CCACCTTAACCTTCCC AATTACTCA	224	58
(2)DAPK1-M	TTAGTTGTGTTTTC GTCGTGTTTC	GCCTTAACCTTCCCAA TTACTCGA	224	57
(4)DAPK1-U	GAGATTGATGTATG AGGGGGTTAT	TACCAAATTCCTCACC AATATCATA	164	55
(4)DAPK1-M	GAGATTGATGTATG AGGGGGTTAC	AATTCCTCGCCGATAT CGTA	159	55
(1)BRCA2-U	AATTATTTGATTTTT GGAGGTGG	ACAAAATTTCACTCTT ATAACCCAAA	87	65
(1)BRCA2-M	GAGAATTATTTGAT TTTTGGAGGC	GAAATTTCGCTCTTAT AACCCG	88	65
(2)BRCA2-U	GTTTTTGAAATTAG GTGGTAGAGGTGG	CAAAAACAAAAAAAC AAAAAACCACAAC	297	55
(2)BRCA2-M	AGTTTTTGAAATTA GGCGGTAGAGGC	AAAAACAAAAAAACA AAAAACCGCG	297	55
(4)BRCA2-U	GATATGTTGATGGG AATTATTAGGTG	ATACCACTAACCACA TTAAACACTCAA	164	61
(4)BRCA2-M	GATATGTTGATGGG AATTATTAGGC	ATACCACTAACCACA TTAAACACTCG	164	61
H19-U	GTTTGGGAGAGTTT GTGAGGTTGT	TCCAATTAACCAAAC TTATACTAATCACCA	187	58
H19-M	GGTTTGGGAGAGTT TGTGAGGTC	CCAATTAACCGAACT TATACTAATCACCG	187	58

1: The number in the ( ) indicates the location of the CpG island. For example: (1) indicates the first CpG island which is located furthest away from the start codon of the gene.

CHARPTER THREE
RESULTS

3.1. Sequence Alignment

In order to design primers at the CpG islands at promoter regions of each gene, it is necessary to find the correct 5' upstream transcription region (UTR) and partial coding region first. Sequences from previous published papers were all based on mRNA sequence from genebank. Published primers for RASSF1A (genebank NM_007182) and DAPK1 (genebank X76104) were initially tried, but the results of MSP were very inconsistent. New primers sets were designed for these two genes as noted below. The first set of primers designed for BRCA2 were based on the genebank DNA sequence (NM_000059), but failed to provide the expected MSP bands. In order to solve this MSP primer design, a new approach to designing MSP primer sets was needed. Genome Browser is a new online genomic database which launched on 2004 with more complete genomic sequences and functions, which could specify the sequence of exon, intron and start codon. UTG and partial coding sequence alignment between genebank and Genome Browser were performed in order to obtain a more accurate sequence for each gene.

Alignment for RASSF1A (Fig 3.1.a) using genebank sequence NM_007182 provided the complete sequence of exon1 on chr3:50342221-50353371. Alignment for RASSF1A (Fig 3.1.a) showed NM_007182 provided the complete sequence of exon1 on chr3:50342221-50353371. Genome Browser shows DAPK has three isomers which are DAPK1, DAPK2 and DAPK3. DAPK1 is located at chr9:87341696-87553100 (CR749834), DAPK2 is located at chr15:61986288-62125574 (NM_014326), and DAPK3

is located at chr19:3909451-3920826 (NM_001348). Previous published papers regarding methylation status of DAPK were based on gene bank X76104. Alignment for X76104 showed that these sequences were actually at chr9 which meant it actually refered to DAPK1. But alignment for DAPK1 showed that UTG sequence from X76104 was split by intronic sequences (Fig3.1.b). Part of the sequence was in the first intron, and the rest of the sequence was in the second exon which resides on chr9:87,341,697-87,553,100. Alignment for BRCA2 sequences also demonstrated that the UTR sequences from NM_000059 are devided on chr13:31787617-31788610. Part of the sequence is on the first exon, while the rest is on the second exon (Fig 3.3c). Sequences for H19 were taken used from Genome Browser, because there was no specific UTR sequence provided by genebank.

(a)

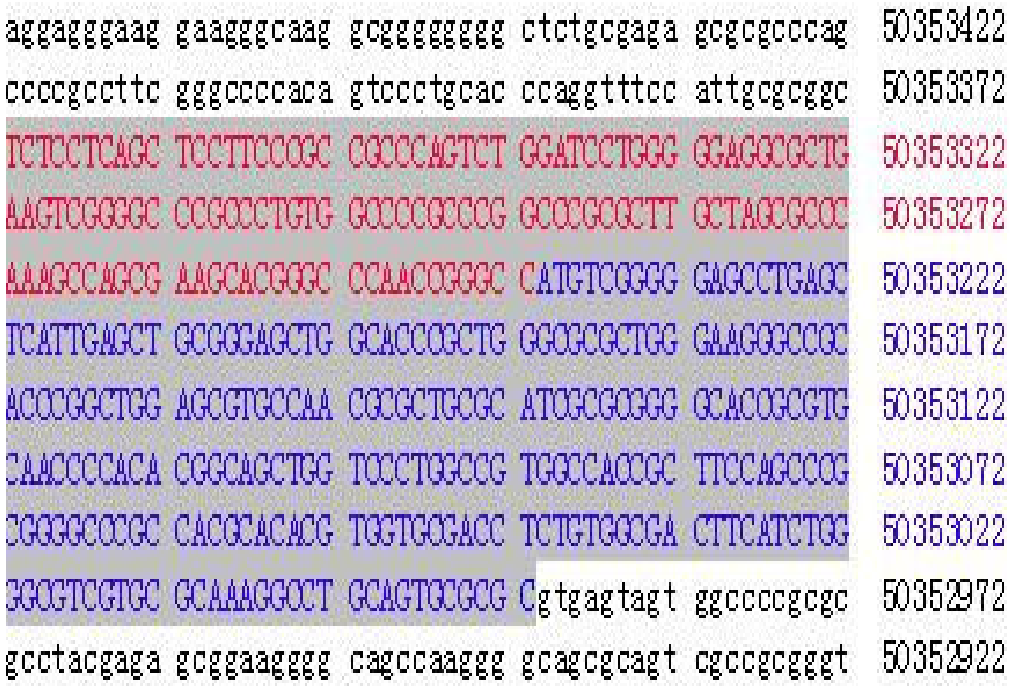

Figure3.1 a: Alignment of RASSF1A.b: Alignment of DAPK1. C: Alignment of BRCA2. Matching bases in coding regions of cDNA from genebank and genomic sequences are colored blue and capitalized. Matching bases in UTR regions of cDNA and genomic sequences are colored red and capitalized. Grey highlighted sequences are exons.

(fig.cont'd)

(b)


ctctgcgtct ctgttgcttc tttgggtctc ggagacctca acccttt ctt 87341699
cagattgcaa accttcttgc cttcaagcct cggctccaac accagtccgg 87341749
cagaggaacc cagtctaatg aggtacgctc ccttcctgcc attctctatt 87341799
ccattaacct gtttcgtggt aaacgtagga ctgatcctcc aaaattacct 87341849
tattaattag cttacatatt tattatctat ctgtcccacc agaatgcagg 87341899
tttccggaag gcagggattt aaaaaaatct gttttgttct atgtgatttt 87341949
cccataccaa gcaccgtgcc cggcacaagc tgggatccca gtacacatct 87341999
cgggacggaa gaaccgtgtt tccctagaac ccagtcagag ggcagcttag 87342049
caatgtgtca caggtggggc gcccgcgttc cgggcggacg cactggctcc 87342099
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ccctccgccc aaaaggcggc aaggagccga gaggctgctt cggagtgtga 87342349
GGAGGACAGC CGGACCGAGC CAACGCCGGG GACTTTGTTC CCTCCgCGGA 87342399
GGGGACTCGG CAACTCCGCAG CGGCAGGGTC TGGGGCCGGC GCCTGGGAGG 87342449
GATCTGCGCC CCCCACTCAC TCCCTAGCTG TGTTCCCGCC GCCGCCCCGG 87342499
CTAGTCTCCG GCGCTGGCGC CTATGGTCGG CCTCCGACAG CGCTCCGGAG 87342549
GGACCGGGGG AGCTCCCAGG CGCCCGGgtg agtagccagg cgcggctccc 87342599
cggtcccccc gacccccggc gccagctttt gctttcccag ccagggcgcg 87342649
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GATGCATGAG GGGgCTACGG AGGCGCAGGA GCGGTGGTGA TGGTCTGGGA 87343499
AGCGGAGCTG AAGTgCCCTG GGCTTTGGTG AGGCGTGACA GTTTATCATG 87343549
ACCGTGTTCA GGCAGGAAAA CGTGGATGAT TACTACGACA CCGGCGAGGA 87343599
ACTTGGCAGg taaaggggt accagaagcg taccctcctg gattgtggaa 87343649

(fig.cont'd)

(c)

```
ccacactgag aaatacccgc agcggcccac ccaggcctga cttccgggtg  31787566
gtgcgtgtgc tgcgtgtcgc gtcacggcgt cacgtggcca gcgcgggctt  31787616
GTGGCGCGAG CTTCTGAAAC TAGGCGGCAG AGGCGGAGCC GCTGTGGCAC  31787666
TGCTGCGCCT CTGCTGCGCC TCGGGTGTCT TTTGCGGCGG TGGGTCGCCG  31787716
CCGGGAGAAG CGTGAGGGGA CAGATTTGTG ACCGGCGCGG TTTTTGTCAG  31787766
CTTACTCCGG CCAAAAAAGA ACTGCACCTC TGGAGCGGgt tagtggtggt  31787816
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tcccgcctcc ggcccggcct ttgggctccg ccttcagctc aagacttaac  31788066
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gatcacttta acggaatatt gctgttttgg ggaagtgttt tacagctgct  31788166
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ctctgtcccc actagccacg cgtcactggt tagcgtgatt gaaactaaat  31788316
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aaacaaattt tccagcgctt ctgagtttta cctcagtcac ataataagga  31788516
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ACCAAGCATT GGAGGAATAT CGTAGGTAAA AATGCCTATT GGATCCAAAG  31788616
AGAGGCCAAC ATTTTTTGAA ATTTTTAAGA CACGCTGCAA CAAAGCAGgt  31788666
attgacaaat tttatataac tttataaatt acaccgagaa agtgttttct  31788716
```

3.2. RASSF1A

Agathanggelou et al (2005) mentioned actually there are two CpG islands are associated with the promoter regions of RASSF1. The first and also the smaller of the two spans the promoter region of RASSF1A (and RASSF1A, RASSF1E, RASSF1F, and RASSF1G). The second CpG island encompasses the promoter regions for RASSF1B and RASSF1C. The entire first exon of each RASSF1 transcript is contained within the CpG islands. Using MethPrimer, two CpG islands were identified in the estimated promoter region and extended regions. The first CpG island was located before the first exon and the second

CpG island expanded from the end of the promoter region into and through Exon1 and partway into Intron1 (Figure 3.2) .The size, start point and end point of each CpG island on the input sequence are listed at table 3.1. Since there are only 8 bp apart, these two islands could be considered as one which is concordance to the smaller one that Agathanggelou *et al.* mentioned.

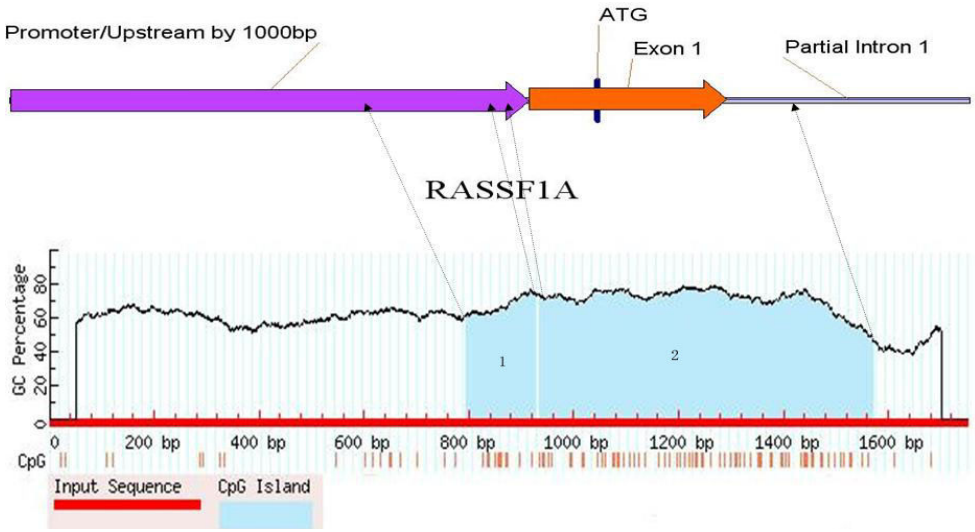

Figure 3.2: The distribution of CpG islands at RASSF1A.

Table 3.1. CpG islands of RASSF1A

	Size(bp)	Start - End
Island 1	133	795 - 927
Island 2	559	935 - 1493

Two sets of primers were designed to study the methylation status of RASSF1A. The first primer sets (both U and M primers) amplified the region across both islands but before the start codon. A total of 8 samples were analyzed and were all found only unmehtylated. However, the bands were not very clear and the results questionable.

The second sets of primers designs were positioned right after the start codon of RASSF1A but within the second CpG island region. A total of 118 samples were analyzed

and the results are listed in table 3.10 and figure 3.3.

Methylation was found in twenty three of fifty two (44.2%) samples of vulvar SCC and eleven of twenty six (42.3%) normal tissues samples (Table 3.2). But there were only seven out of forty (17.5%) lichen sclerosis tissues. The methylation of SCC specimens using the RASSF1A primer was found to be significantly higher than lichen sclerosis tissues (p=0.0067), but not significantly different from normal tissue (p=0.8717). Lichen sclerosis was also significantly less methylated than Normal tissue (p=0.0270).

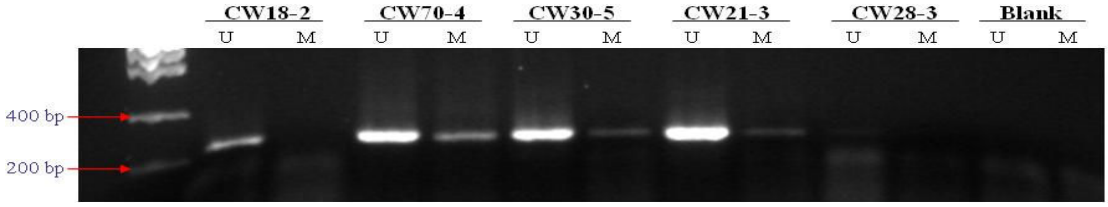

Figure3.3 Representative samples of MSP analysis of the RASSF1A gene on the 2nd CpG island

Table 3.2. The methylation status for RASSF1A

	Unmethylated	Methylated	Total	Methylation (%)
Normal	15	11	26	42.3
LS	33	7	40	17.5
SCC	29	23	52	44.2

3.3. DAPK1

Four CpG Islands were found in the DAPK1 gene sequence with the Methprimer software. The first CpG island is located in the promoter region. The second and the third CpG island are both located within the first inron. The fourth CpG island extends from the end of Inron1 to almost the end of Exon2 (Figure 3.4). The size, start point and end point of each CpG island are listed at table 3.3.

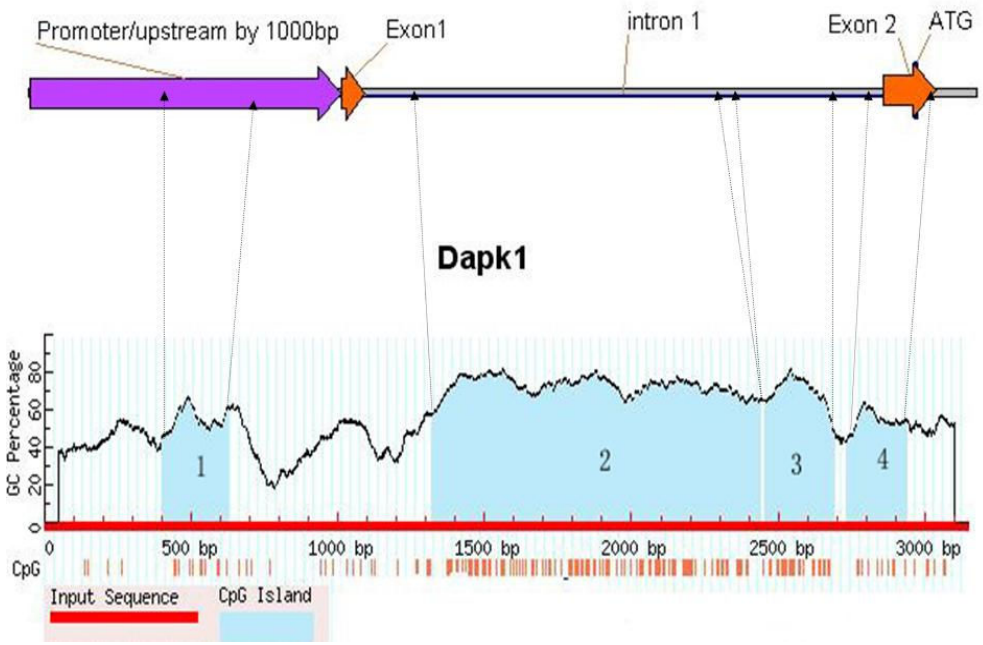

Figure3.4: The distribution of CpG islands at Dapk1

Table3.3. The distribution of CpG islands of DAPK1

	Size(bp)	Start - End
Island 1	224	403 - 626
Island 2	1119	1322 - 2440
Island 3	238	2456 - 2693
Island 4	206	2456 - 2693

Three sets of primers were designed for studying the methylation status of DAPK1 within the first, second and fourth CpG islands. Eight samples were analyzed for the first CpG island region, and twenty samples were analyzed for the fourth CpG island region. All of the samples showed both bands for unmethylation and methylation on both regions (Figure 3.5).

A total of 107 samples were analyzed by MSP on the second CpG island (Table 3.10, Figure 3.5). From table 3.4, hypermethylation was found in twenty three of fourty four (52.3%) samples of vulvar SCC and eighteen of thirty seven (48.7%) lichen sclerosis samples. But there were only six out of twenty six (23.0%) normal specimens found to be

hypermethylated. The methylation of SCC specimens was found to be significantly higher than adjacent normal tissues (p=0.0166), as was lichen sclerosis tissues compared to normal tissues (p=0.0396). However, the occurrence of methylation in SCC samples was not significantly different from lichen sclerosis samples (p=0.7452).

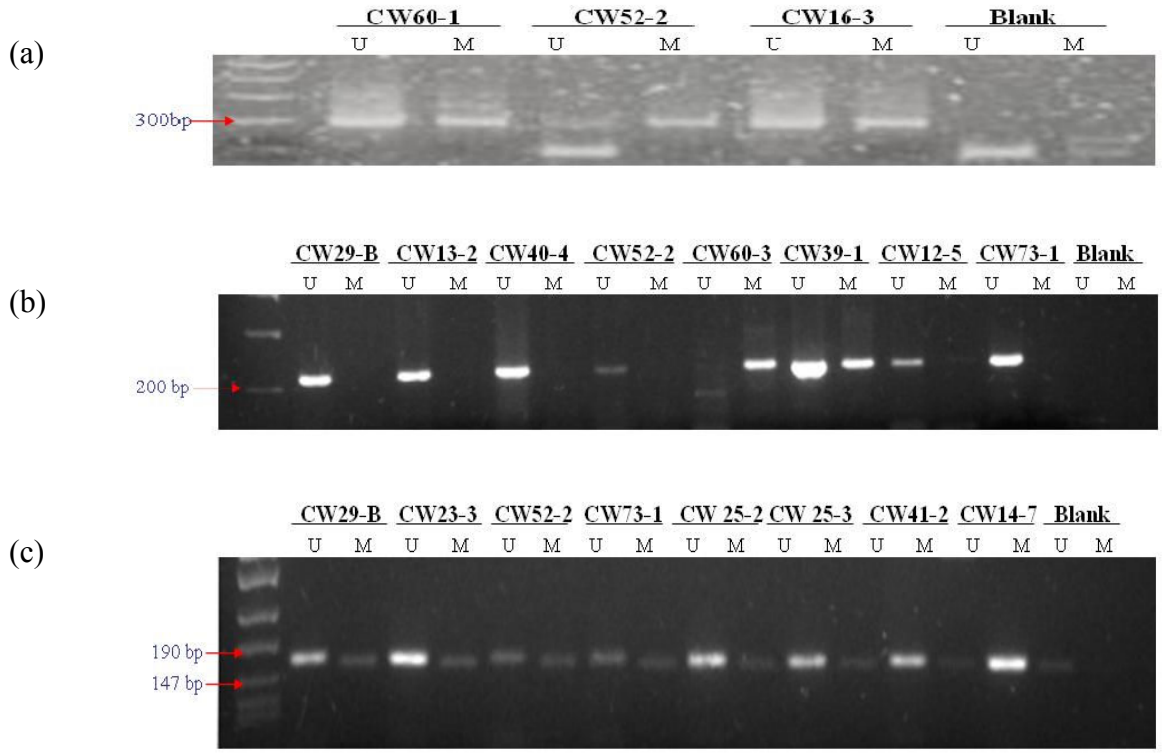

Figure 3.5 Representative samples of MSP analysis of the DAPK-1 gene. location: a)1st CpG island, b)2nd CpG island, c)4th CpG island

Table 3.4. The methylation status of the second CpG island of DAPK1

	Unmethylated	Methylated	Total	Methylation (%)
Normal	20	6	26	23.0
LS	19	18	37	48.7
SCC	21	23	44	52.3

3.4. BRCA2

Four CpG Islands were found in BRCA2 sequence using the Methprimer program (Figure 3.6). The first CpG island is located in the promoter region and the second CpG island extended from the end of the promoter region into and through Exon1 and partway

into Intron1. The third and the fourth CpG islands are both located within Intron 1 (Figure 3.4). The size, start point and end point of each CpG island are listed at Table 3.5.

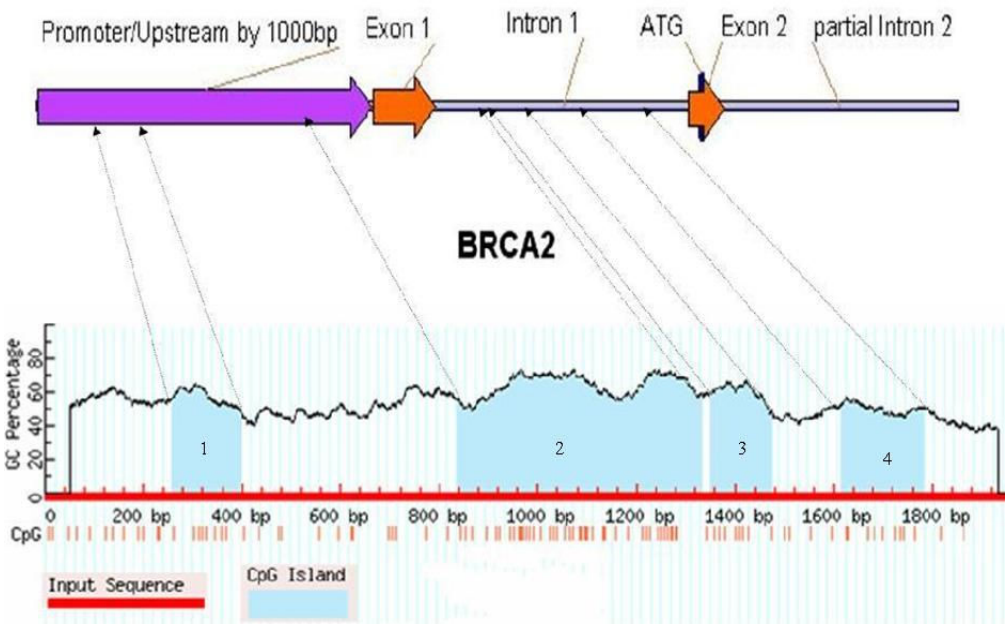

Figure3.6. The distribution of CpG islands at BRCA2

Table3.5. Distribution of CpG islands of BRCA2

	Size(bp)	Start - End
Island 1	140	259 - 398
Island 2	496	836 - 1331
Island 3	127	1349 - 1475
Island 4	166	1616 - 1781

Three sets of primer sets were designed to study the methylation status of BRCA2 within the first, second and fourth CpG islands. Seven samples were analyzed for the first CpG island region, and twenty three samples were analyzed for the fourth CpG island region. All of the samples showed both unmethylation and methylation bands on both the first and fourth CpG regions (Table 3.10, Figure 3.7). Fifteen samples for the second CpG island region showed only unmethylation (Table 3.10, Figure 3.7).

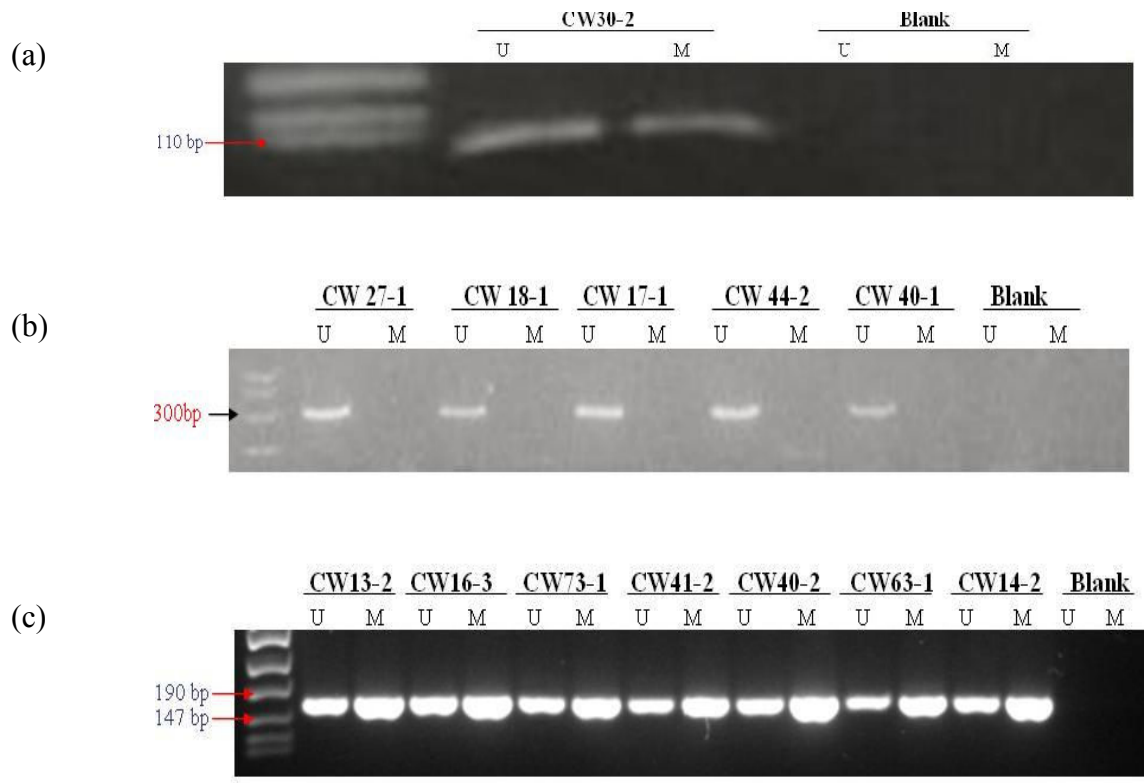

Figure 3.7 Representative samples of MSP analysis of the BRCA2 gene. location: a)1st CpG island, b)2nd CpG island, c)4th CpG island

Since regular MSP could not detect changes in the methylation level of Brca2, real-time PCR was performed using the fourth CpG island primer set. Three replicates of each sample for both U and M PCR were performed.

Dissociation curves starting at 60 ^{o}C were performed after 40 cycles to ensure there were no non-specific PCR products. A single product would show a single sharp peak at its melting temperature, but non-specific products such as primer dimers would show another sharp peak at its own melting temperature (Figure 3.8). Dissociation curves depend on PCR products' GC content, size etc. Therefore, even two products with same size but different GC content could be differentiated by dissociation cures which could not be seperated by regular electrophoresis. The determination of dissociation curves is a necessary and powerful step which should be included in SYBR-green real-time PCR.

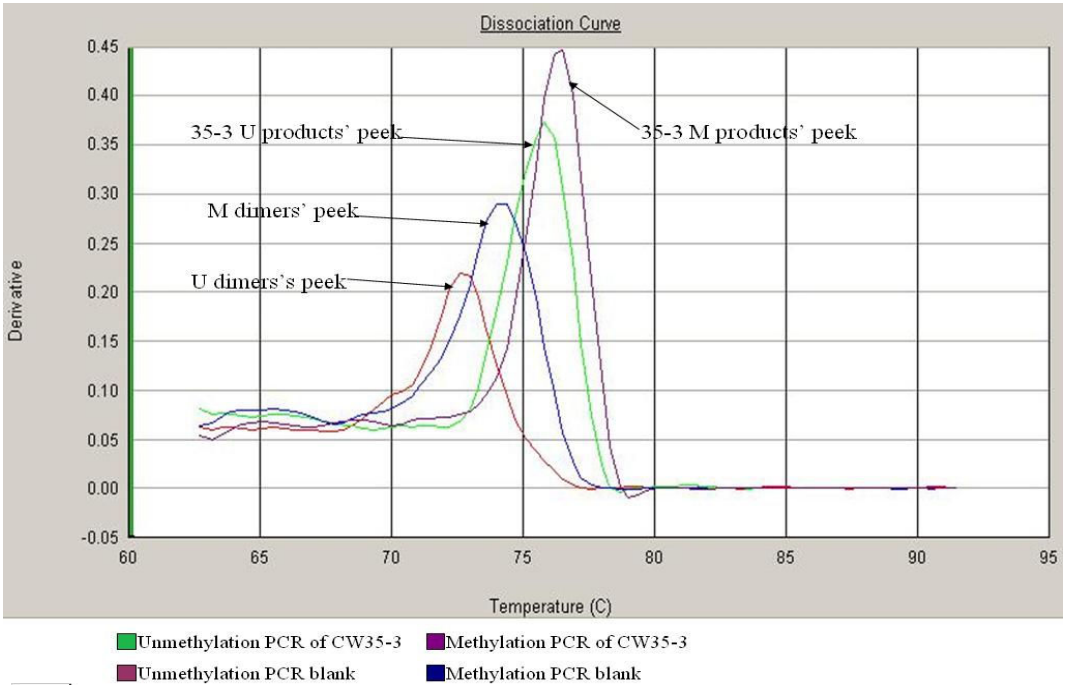

Figure 3.8. Dissociation curve for one replicate of sample 35-3 and blank

Twenty five samples were analyzed by real-time PCR using the same primer sets as regular MS-PCR (Table 2.1).Only fifteen samples gave valid data due to the presence of non-specific products (Table 3.9). Primer dimers tended to form when the initial concentrations of DNA templates were low. For example, sample CW 70-5 (Figure 3.9), all three replicates of the U reaction showed products and dimers, while only pure M Products were observed in the same sample. This phenomena could be because there were not enough unmethylated templates in the sample due to hypermethylation. Other samples had dimers in both U and M reactions, which might because there were not enough templates left after bisulfite treatment.

As mentioned in Methods, C_t value is the cycle number when the fluorescence light produce by SYBR-green attached to products reached the threshold. The greater the initial number of templates, the earlier the product fluorescence light would reach to the threshold. The difference of the C_t value (D_{ct}) between U and M PCR reactions from each sample would

indicate if the methylation level of the original copy number increases or decreases (Figure 3.10).

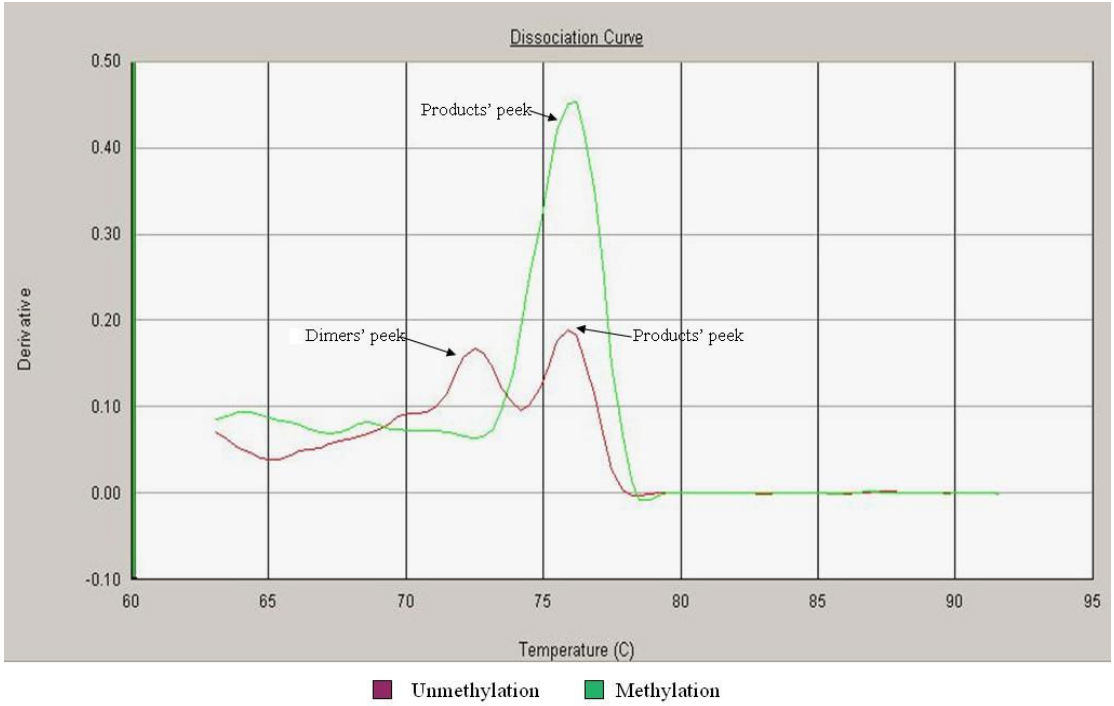

Figure 3.9 Dissociation curve for one replicate of sample 70-5

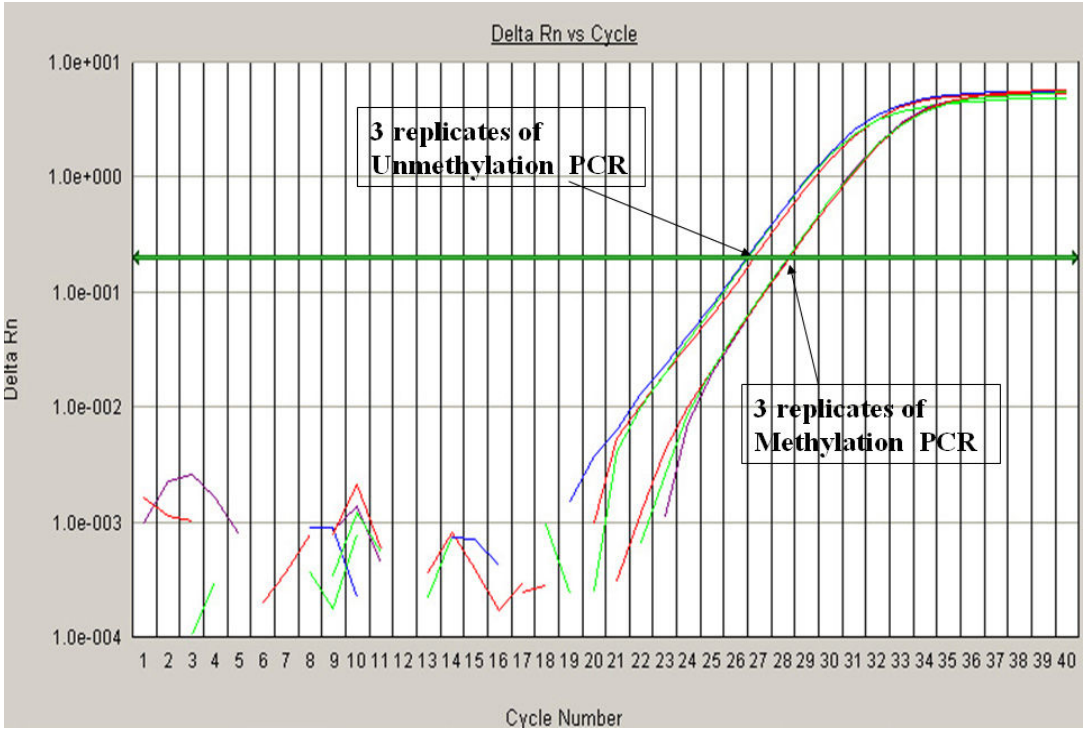

Figure 3.10 Applification plot of sample CW 35-3.The Green line is the threshold, and the cycle number that the Delta Rn reaches the threshold is the Ct Value for that reaction.

Since the amount of fluorescence light is proportional to the total amount of the product, and the PCR product size of U and M are same. Therefore, if we assume the reaction efficiencies for both sets of primers are same, we can give an estimation of the proportion of the initial unmethylated templates and methylated templates in a heterozygous cell. Using U_i to represent the percentage of initial amount of unmethylated templates, M_i to represent the percentage of initial amount of methylated templates, a and b represent the C_t value for U reaction and M reaction respectively. By the time both reaction reached to threshold, the total templates in each reaction should follow equation below:

$$\boldsymbol{U_i} * 2^{a} = \boldsymbol{M_i} * 2^{b} \qquad \text{(Equation 3.1)}$$

$$\boldsymbol{U_i} + \boldsymbol{M_i} = 100 \qquad \text{(Equation 3.2)}$$

Therefore, we can find that the proportion of methylated templates is :

$$\boldsymbol{M_i} = \frac{100}{2^{(b-a)} + 1} \qquad \text{(Equation 3.3)}$$

Based on equation 3.3, we can estimate how much percentage of methylated templates and whether it increases or decreases in each sample.

Three replicates of each sample were performed, but not every replicate gave a specific product. Only data from those replicates that showed no non-specific products were used. However, it was necessary to use at least two replicates for each sample to ensure accuracy. Therefore, only fifteen samples had at least two replicates with pure products. Also, due to experiment error, even those samples with all three replicates having specific products did not have similar C_t values. Sometimes one of the C_t values would be different from the other two replicates' values. In this situation, only the two C_t values close to each other were chosen. Means of C_t values, D_{ct} values and M values of all the replicates for each sample and each

type of reaction were calculated for final analysis (Table 3.6)

Table 3.6.The average values of each sample

Sample	tissue type	Average Ct (M)	Average Ct (U)	Average D_{ct}	Average % of U	Average % of M
14-7	N	26.0933	24.4700	1.6233	75.4954	24.5046
2b	N	23.9933	22.4033	1.59	75.0658	24.9342
30-2	N	25.1767	22.8633	2.3133	83.2507	16.7493
39-1	N	24.3733	22.2900	2.0833	80.9082	19.0918
40-4	N	26.0700	23.0867	2.9833	88.774	11.226
41-2	N	25.3467	22.735	2.6117	85.9394	14.0606
52-2	N	31.4350	30.0250	1.41	72.658	27.342
52-1	LS	25.1033	24.06	1.0433	67.3308	32.6692
52-6	LS	25.2400	23.8800	1.36	71.9641	28.0359
27-1	SCC	24.2400	22.9033	1.3367	71.6371	28.3629
35-3	SCC	28.3867	26.7633	1.6233	75.4958	24.5042
50-4	SCC	32.0833	30.6300	1.4533	73.2506	26.7494
50-7	SCC	30.9300	29.1400	1.79	77.5692	22.4308
63-1	SCC	28.7700	27.2100	1.56	74.6742	25.3258
91-2	SCC	26.5167	25.2550	1.2617	70.5685	29.4315

Least Square Means were calculated for the percentage of mehylation templates (M) and the differences of the C_t values (D_{ct}) were based on the tissue type (Table 3.7). ANOVA analysis performed for both M and D_{ct} based on the tissue type, and comparison between each type of tissue were also performed (Table3.8, table 3.9)

Table 3.7 Estimated Least Squares Means of D_{ct} and M

Tissuetype	Estimated D_{ct}	Standard Error (D_{ct})	Estimated M	Standard Error (M)
N	2.0879	0.1651	19.7012	1.7780
LS	1.2017	0.3088	30.3526	3.3263
SCC	1.5042	0.1783	26.1341	1.9205

Table 3.8 Differences of Least Squares Means of Dct

Effect	Tissuetype-Tissuetype	Estimate	Standard Error	Df	t Value	Pr>|t|
tissuetype	LS_N	-0.8862	0.3502	12	-2.53	0.0264
tissuetype	LS_SCC	-0.3025	0.3566	12	-0.85	0.4129
tissuetype	N_SCC	0.5837	0.243	12	2.4	0.0334

Table 3.9 Differences of Least Squares Means of percentage of methylation

Effect	Tissuetype-Tissuetype	Estimate	Standard Error	Df	t Value	Pr>|t|
tissuetype	LS_N	10.6513	3.7717	12	2.82	0.0153
tissuetype	LS_SCC	4.2185	3.8409	12	1.1	0.2936
tissuetype	N_SCC	-6.4329	2.6171	12	-2.46	0.0301

The results demonstrates that the value of D_{ct} and percentage of methylated templates (M) varied based on tissue type. The mean D_{ct} value of normal tissues was significantly higher than that of lichen sclerosis (LS) tissue (p=0.0264), and that of SCC (p=0.0334), but the mean D_{ct} values between SCC and LS were not significantly different (p=0.4129). The percentage of methylated templates of normal tissue was significantly less than that of LS (p=0.0153), and less than that of SCC (p=0.0301), but SCC and LS were not significantly different (p=0.2936).

Real-time PCR was also performed at the first CpG island by using the same primers for MSP listed at table 2.1, but no accurate results were collected since dissociation cures indicated non-specific products constantly existed at the methylation PCR reaction no matter what conditions were used. Gel electrophoresis also verified that primer dimmers were the consistent non-specific products which interfered the results.

3.5 H19

H19 is located on chr11:1,972,984-1,975,280. Only one CpG island was found in the gene sequence using the Methprimer program. The size, start point and end point of the CpG island at input sequence are listed at table 3.8, shown in figure 3.11.

H19 is a maternal imprinted gene. Seven samples were tested with MSP and both U and M bands were displayed in all cases. Therefore, real-time PCR was tested for the detection of a change in each sample. Three different sets of primers were designed and tested in

real-time PCR (Figure3.12). Unfortunately, dissociation curves showed there were consistant primer dimmers in the products from every set of primers. Therefore, real-time PCR with SYBR-green as reporter dye failed to detect changes in the methylation level for H19.

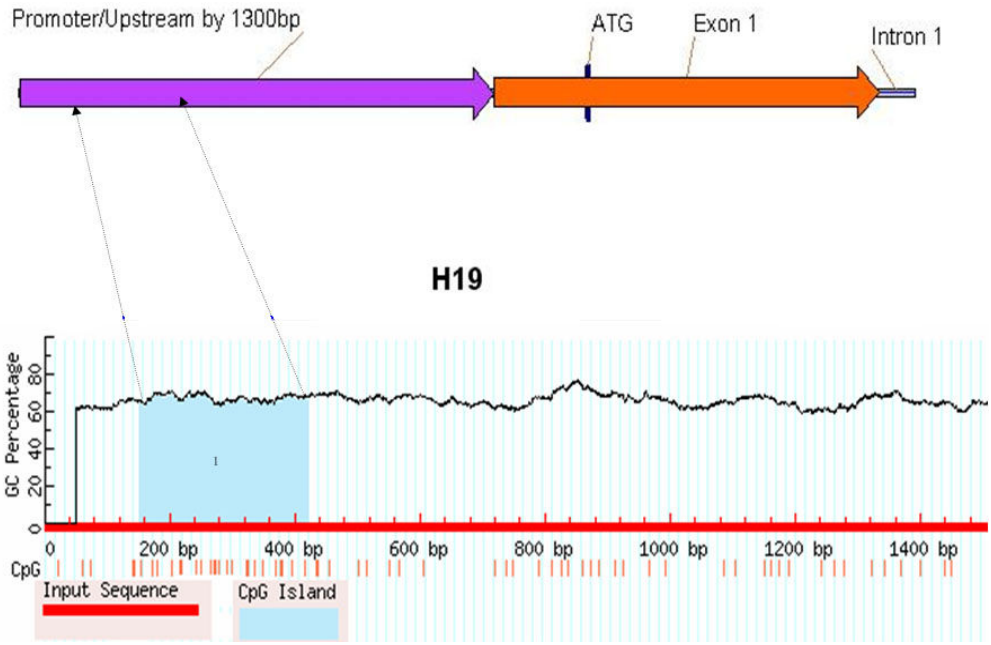

Figure3.11. The distribution of CpG islands at H19

Table 3.8. CpG islands of H19

	Size(bp)	Start - End
Island 1	133	795 - 927

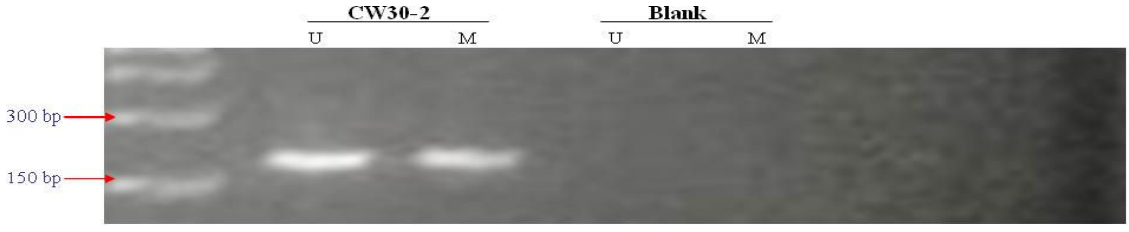

Figure 3.12 Representative samples of MSP analysis of the H19 gene from one primer sets.

Table 3.9 Real-time PCR data of BRCA2

Sample	PCR type	TissueType	Ct Value
35-3	U	SCC	27
35-3	U	SCC	26.66
35-3	U	SCC	26.63
35-3	M	SCC	28.36
35-3	M	SCC	28.44
35-3	M	SCC	28.36
52-1	U	LS	24.07
52-1	U	LS	24.04
52-1	U	LS	24.07
52-1	M	LS	25.11
52-1	M	LS	25.02
52-1	M	LS	25.18
14-7	U	N	24.5
14-7	U	N	24.44
14-7	M	N	26.17
14-7	M	N	26.02
14-7	M	N	26.09
52-6	U	LS	23.78
52-6	U	LS	23.87
52-6	U	LS	23.99
52-6	M	LS	25.14
52-6	M	LS	25.27
52-6	M	LS	25.31
91-2	U	SCC	25.14
91-2	U	SCC	25.37
91-2	M	SCC	26.43
91-2	M	SCC	26.48
91-2	M	SCC	26.64
2b	U	N	22.51
2b	U	N	22.25
2b	U	N	22.45
2b	M	N	23.97
2b	M	N	23.9
2b	M	N	24.11
30-2	U	N	22.93
30-2	U	N	22.88
30-2	U	N	22.78
30-2	M	N	25.16
30-2	M	N	25.13
30-2	M	N	25.24
41-2	U	N	22.43
41-2	U	N	23.04
41-2	M	N	25.44
41-2	M	N	25.38
41-2	M	N	25.22

(TABLE continued)

27-1	U	SCC	22.9
27-1	U	SCC	22.85
27-1	U	SCC	22.96
27-1	M	SCC	24.31
27-1	M	SCC	24.2
27-1	M	SCC	24.21
50-4	U	SCC	30.98
50-4	U	SCC	30.28
50-4	M	SCC	31.48
50-4	M	SCC	32.69
50-4	M	SCC	32.08
50-7	U	SCC	29.14
50-7	U	SCC	29.14
50-7	M	SCC	30.78
50-7	M	SCC	31.08
63-1	U	SCC	26.89
63-1	U	SCC	27.44
63-1	U	SCC	27.3
63-1	M	SCC	29.1
63-1	M	SCC	28.64
63-1	M	SCC	28.57
39-1	U	N	21.93
39-1	U	N	22.65
39-1	M	N	24.41
39-1	M	N	24.19
39-1	M	N	24.52
40-4	U	N	22.77
40-4	U	N	23.25
40-4	U	N	23.24
40-4	M	N	26.02
40-4	M	N	26.1
40-4	M	N	26.09
52-2	U	N	29.81
52-2	U	N	30.24
52-2	M	N	31.51
52-2	M	N	31.36

Table 3.10 MSP analysis of each sample

Sample	Type	RASSF1A		DAPK1			BRCA2			H19
		1st CpG	2nd CpG	1st CpG	2nd CpG	4th CpG	1st CpG	2nd CpG	4th CpG	1st CpG
CW 5-4	LS									
CW 5-5	LS									
CW 7-1	SCC									
CW 10-1	SCC									
CW 11-1	SCC									
CW 12-1	SCC									
CW 12-2	SCC									
CW 12-4	SCC									
CW 13-2	N									
CW 13-4	N									
CW 14-1	SCC									
CW 14-2	SCC									
CW 14-7	N									
CW 16-2	SCC									
CW 16-3	N									
CW 17-1	SCC									
CW 17-5	N									
CW 17-6	LS									
CW 18-1	SCC									
CW 18-2	SCC									
CW 18-5	N									
CW 18-6	SCC									
CW21-1	SCC									
CW 21-3	LS									
CW 22-1	SCC									
CW 22-2	LS									
CW 22-3	LS									
CW 22-4	LS									
CW 23-1	SCC									
CW 23-3	N									
CW 24-1	SCC									
CW 25-1	SCC									
CW 25-2	N									
CW 26-2	SCC									
CW 27-1	SCC									
CW 28-1	SCC									
CW 28-3	LS									
CW 28-5	LS									
CW 28-B	N									
CW 29-1	SCC									
CW 29-3	N									
CW 29-4	LS									

(TABLE continued)

CW 29-5	LS									
CW 29-6	SCC									
CW 29-B	N									
CW 30-1	SCC									
CW 30-2	N									
CW 30-3	LS									
CW 30-5	LS									
CW 31-2	LS									
CW 31-3	LS									
CW 35-2	LS									
CW 35-3	SCC									
CW 35-5	LS									
CW 35-6	LS									
CW 39-1	N									
CW 39-3	N									
CW 40-1	SCC									
CW 40-2	SCC									
CW 40-4	N									
CW 41-1	SCC									
CW 41-2	N									
CW 43-1	SCC									
CW 43-3	SCC									
CW 43-4	SCC									
CW 44-1	N									
CW 44-2	SCC									
CW 44-3	LS									
CW 44-4	LS									
CW 44-5	LS									
CW 44-7	LS									
CW 45-4	N									
CW 45-5	LS									
CW 46-3	LS									
CW 46-4	LS									
CW 46-5	LS									
CW 46-6	LS									
CW 46-7	LS									
CW 47-2	SCC									
CW 47-3	LS									
CW 47-4	LS									
CW 49-1	SCC									
CW 49-3	SCC									
CW 49-4	LS									
CW 49-5	LS									
CW 49-6	LS									
CW 50-1	SCC									
CW 50-2	SCC									
CW50-4	SCC									

(TABLE continued)

CW 50-5	LS									
CW 50-6	SCC									
CW 50-7	SCC									
CW 52-1	LS									
CW 52-2	N									
CW 52-6	LS									
CW 53-1	LS									
CW 53-2	LS									
CW 53-3	LS									
CW 57-1	SCC									
CW 57-3	N									
CW 60-1	N									
CW 60-3	N									
CW 61-1	SCC									
CW 61-2	LS									
CW 61-4	LS									
CW 61-6	SCC									
CW 62-1	N									
CW 63-1	SCC									
CW 63-2	SCC									
CW 63-5	SCC									
CW 64-3	SCC									
CW 66-3	LS									
CW 66-4	LS									
CW 70-1	LS									
CW 70-2	SCC									
CW 70-3	SCC									
CW 70-4	LS									
CW 70-5	LS									
CW 70-6	LS									
CW 73-1	N									
CW 89-1	SCC									
CW 89-2	SCC									
CW 91-1	SCC									
CW 91-2	SCC									
CW 93-1	SCC									
CW 93-2	SCC									
CW 97-1	SCC									
CW 98-1	SCC									
CW 98-4	SCC									
CW 106-3	N									
CW 108-1	N									

Black cell: Sample both methylated and unmethylated

Grey cell: Sample only unmethylated

CHAPTER FOUR
DISSCUSSION

4.1 General Discussion

The promoter regions of four genes have been studied for the methylation pattern in normal vulva tissue to Lichen Sclerosis and Squamous Cell Carcinoma. Since the study region for RASSF1A was designed after the transcription start codon, an interesting methylation pattern was observed that was different from the majority of published results (Burbee et al. 2001; Kim et al. 2003; Kuroki et al. 2003; Wang et al. 2004). The frequency of methylation in RASSF1A significantly decreased (p=0.0270) from normal to LS, then dramatically increase (p=0.0067) from LS to SCC. DAPK-1 was found to be significantly hypermethylated in LS and SCC tissues, which is similar to previous studies on other cancers (Katzenellenbogen et al. 1999; Lehmann et al. 2002; Tada et al. 2002). BRCA2 was found to be hypermentylated in one of its CpG islands in both LS and SCC tissues. In the analysis of the H19 gene, an imprinted gene, real-time PCR failed to detect changes in methylation in diseased specimens. These results demonstrated that different methylation patterns of RASSF1A, DAPK-1 and BRCA2 genes were detected based on different vulva specimen disease states, and indicate that epigenetic events might be involved in or associated with preneoplastic and early tumorigenesis.

4.2 RASSF1A

Although more than 90% of published papers listed on Pubmed have reported that RASSF1A promoter hypermethyltion was associated with reduced mRNA expression in tumors, the present studies display quite different results from previous studies. The

frequency of hypermethylation of RASSF1A was found to be the same in vulva SCC and normal tissue (44.2% and 42.3%, respectively). However, vulvar Lichen sclerosis (LS) tissue only showed 17.5% methylation frequency. Methylation frequency significantly decreased (p=0.027) from normal tissue to LS, and significantly increased (p=0.0067) from LS to SCC. But there is no statistic significance between the Normal and SCC.

As shown in Figure 3.2, the CpG island studied spans the 5'UTG region through first exon and extends past the transcription start codon. Since previous reports focused on only the 5'UTG region, the present results help clarify progressive changes in methylation of developing skin SCC. The majority of published reports used primers located within the CpG island located before the transcription start site. Primers for MSP which amplify the region starting from the end the 5' UTR and passing first exon but before the transcription start codon were published by Burbee et al. (2001), and have been frequently used by other papers. For examples, Kuroki et al. (2003) found hypermethylation in esophageal squamous cell carcinoma sample (52%) vs. only 4% in noncancerous tissues; Kim et al. (2003) reported hypermethylation occurred 26% of lung squamous cell carcinoma and Wang et al. (2004) reported hypermethylation was detected in 39% non-small cell lung cancer tissue and only 3% in corresponding normal appearing lung tissues.

Methylation analyses of the CpG island located after the transcription start site of RASSF1A has however also been reported. Spugnardi et al. (2003) assessed two different regions of the RASSF1A CpG island located on both sides of transcription start codon to study aberrant methylation. Region 1 was located upstream of the transcription start codon and contained three Sp1 consensus binding sites, and the primers for the MSP were similar

to those reported by Burbee et al. (2001). Region 2 was located within the first exon immediately after the transcription start codon which is specific to the RASSF1A transcript and similar to the current studied region. Hypermethylation of RASSF1A region 1 was found in 41% of melanoma tumors, of which 33% of stage III melanoma patients, and 44% of stage IV patients. Analysis of RASSF1A region 2 revealed aberrant methylation in 50% of melanoma tumors with only 22% of stage III patients, but 56% of stage IV patients. There was an increasing frequency of methylation through the tumor passage on region 2, which is similar to my results showing the methylation frequency increase dramatically from LS to SCC (17.5% to 42.3%). No methylation was detected in available normal skin tissues or healthy donor peripheral lymphocytes, but Spugnardi et al. (2003) did not clarify whether the normal tissues were normal adjacent tissue or normal non-adjacent tissue. 64% and 82% of melanoma cell lines have been reported to be methylated in region1and region 2, respectively (Spugnardi et al. 2003). Hypermethylation was higher in RASSF1A region 2 in both cell lines and tumors, and RT-PCR revealed that samples where both regions were methylated lacked RASSF1A gene expression (Spugnardi et al. 2003). Although at present, the mechanisms of de novo methylation of tumor suppressor genes and other regulatory genes are poorly understood, Spugnardi *et al.* proposed that it is possible that the hypermethylation event initiated in region 2 (exon 1) and then spread into region 1, the upstream promoter region, which ultimately results in gene silencing.

Strunnikova et al. (2005) also investigated the methylation pattern of Human mammary epithelial cells (HMEC) in different regions of the RASSF1A gene including a region similar to that reported by Spugnardi et al. (2003) which contained three Sp1

binding sites and the transcription start site (RA region), an upstream region to RA (U1) and a downstream regions (D1). All of these regions were unmethylated in normal human fibroblasts, blood leukocytes, and Hela cells. The RA region was completely unmethylated in prestasis and stasis HMEC, and only methylated in postasis cells. Stasis is the state, in which the normal flow of a body liquid stops, for example the flow of blood through vessels or of intestinal contents through the digestive tract (Wikipedia contributors 2005). However, both U1 and D1 which flanked the transcription initiation site exhibited frequent methylation along with further increase in methylation in later passages of HMEC cultures. RASSF1A was also dramatically silenced with increasing passages. My work has shown that methylation dramatically increased from LS to SCC. My work also found that normal tissues were also highly methylated. Unfortunatley, Strunnikova et al. (2005) did not report the methylation pattern in the normal tissues. Based on the published results, Strunnikova et al. (2005) also proposed there might be a spreading of de novo DNA methylation from the methylated upstream and downstream regions into the RASSF1A CpG island promoter.

It appears that the primary control of gene expression may be due to methylation status of the CpG island in the RASSF1A promoter region. However, understanding the progressive changes in RASSF1A gene methylation in cancer development may require the analysis of CpG islands before and after the start codon. The region that right before the start codon which contains three Sp1 sites is frequently hypermethylated in human tumors but unmethylated in normal tissues, while the down stream region is more frequently unmethylated in normal tissue and the methylation increases with tumor development.

Current studies show that the methylation frequency significantly increases from LS to

SCC at the down stream region, which is in concordance with previous studies, but it is hard to explain that even normal tissues were as frequently methylated as SCC. And it is also questionable that the de novo methylation spreading hypothesis proposed by Spugnardi *et al.* and Strunnikova et al is valid.

4.3 DAPK-1

At the first and fourth CpG islands in the 5' region of the DAPK-1 gene sequences, MSP results showed both unmethylated and methylated bands were consistantly present in all samples analyzed. This indicated that both alleles at these two regions might be heterozygous by methylation. However, the second CpG island showed variation of methylation patterns in different samples. Relating this information with Figure 3.3, it is quite obvious that 5' upstream region, and the regions slightly before exon2 might not be involved in epigenetic events associated with gene expression process, while the first intron is probably involved.

Hypermethylation of CpG islands in DAPK-1 has been frequently observed in various types of human malignancies. The methylation results observed for DAPK-1 in the present study were consistant with those seen in other studies. This study demonstrated DAPK-1 was found to exhibit a high frequency of promoter methylation in vulvar Lichen sclerosis (LS) and SCC. These values for SCC and LS tissues were both found to be statistically different from normal tissues. This relationship showing increasing methylation from normal to LS and SCC suggests that epigenetic silencing of DAPK-1 is an early event in vulvar neoplasia. These data correlate well with data from others studying SCC and other cancers such as 53% in lobular invasive breast cancer, 9% in ductal invasive breast cancer,

76.1% in nasopharyngeal carcinoma (NPC), 34%-35% in lung SCC, 29% in epithelium bladder carcinoma and 84% in B-cell non-Hodgkin's lymphomas, (Katzenellenbogen et al. 1999; Chan et al. 2002; Lehmann et al. 2002; Tada et al. 2002; Mittag et al. 2005)

4.4 BRCA2

Few papers about the study of aberrant methylation of the BRCA2 gene have been published. Hilton et al. (2002) has reported that only 1 out of 12 (8%) tumors in ovarian cancers lacked detectable BRCA2 mRNA showed hypermethylation, but all eight of the tumors without detectable BRCA1 mRNA demonstrated BRCA1 promoter CpG island hypermethylation. Dhillon et al. (2004) also reported a similar low occurrence of BRCA2 methylation in human primary tumors with only 1 out of 25 (4%) Granulosa cell tumor cell lines hypermethylated. Both of these papers used the same primer sets for MSP, which were located on the second CpG island as shown on in Figure 3.4, but neither of them reported the methylation pattern in the normal tissues. These two papers also hypothesized that the methylation pattern might not contribute to gene expression mechanism, although other epigenetic events might be involved. It is possible that the region they studied might not be involved in epigenetic instability, which also means it might not be the active promoter region of BRCA2.

The second CpG island in the BRCA2 gene sequence extends from 5' UTR, across the exon1 and all the way down to intron1. Since the third island is only 18bp apart from the second one, it was grouped together with the second CpG island. Only unmethylated MSP patterns were observed at this region, suggesting that both alleles might be homozygous unmethylated at this locus.

The first CpG island is located in the 5' UTR, while the fourth CpG island is in the first intron but close to the transcription start codon. Both unmethylated and methylated patterns were shown by MSP at the these two islands 100% of the time in vulva specimens. However, partial variation of the methylation pattern could not be detected by regular MSP at these two CpG islands. Real-time PCR revealed that the percentage of copy numbers of methylated alleles statistically significantly increased from normal tissue (19.7%) to LS (30.4%) and SCC (26.1%), but not between LS and SCC. If we assume both U and M primer sets have the same reaction efficiencies, then the results would indicate that there exists an increase of methylation of BRCA2 in the LS and SCC tissue specimens compared to adjacent normal vulva tissue.

Another assumption is that the primer sets' efficiency causes the difference of Ct value's which biased the data increasing the determined methylated templates in the cancer tissues. However, from the results, approximately 80.3% of the alleles were unmethylated in the normal tissue, but only 19.7% were methylated. It is less likely that this 60.3% difference can be explained by the difference between primer sets' efficiency.

Overall, from current studies, it appears that the fourth CpG island of BRCA2 is frequently methylated in cancer tissue. Although inactivation of BRCA2 through promoter hypermethylation has not been reported so far and gene expression level of BRCA2 has not been tested in these vulva samples in the current studies, the change of hypermethylation patterns still could be a molecular maker for the early stage occurrence of neoplaisa.

There is only one paper reporting the loss of methylation in BRCA2. Chan et al. (2002) has reported a significant correlation between hypomethylation and overexpression of

BRCA2. Using enzyme-digested bisufite PCR inclusive of 31 CpG dinucleotides within the promoter and 5'-UTR regions, hypomethylation of BRCA2 was observed in 16 out of 23 cases of sporadic ovarian cancer than in nontumor samples. Unfortunately, the exact location of these CpG sites was not reported. Therefore, it is hard to locate the CpG sites in the promoter region and compare their results with mine.

The current real-time PCR approach to these studies was unable to distinguish any differences in methylation patterns on the first CpG island. But it is still important to find out if any changes in methylation in the first CpG island correlate with changes at the fourth CpG island. Gene expression analysis also needs to be done in order to clarify the correlation between gene expression and methylation pattern in BRCA2.

4.5 H19

H19 is a maternally imprinted gene, and MSP results showed both unmethylation and methylation bands for all the specimens studied, further supporting the heterozygosity of the two alleles in this imprinted gene. Real-time PCR also failed to differentiate changes in the methylation status of H19 in these studies. H19 might be a good indicator sequence if variation in methylation patterns could be detected.

4.6 Real-time PCR

SYBR-green is a less expensive reagent and more easily used for real time PCR than other approaches such as TaqMan and probes. But since it also reports the non-specific products along with the amount of final products, the requirements for the primers' efficiency are very high. Low amounts of template could also cause false negative results which make it is hard to detect the copy number of unmethylated or methylated templates

when hyper- or hypo-methylation has occurred.

TaqMan is another type of fluorescent reporter method commonly used in Real- time PCR. With a matched probe, TaqMan only reports the specific amplicon even in the presence of non-specific products. Based on my experience, it may be necessary to use TaqMan for future studies in order to detect differences between the template copy numbers of unmethylated and methylated templates in target genes.

4.7 Conclusion and Future Research

Although aberrant promoter hypermethylation and hypomethylation phenomenon in tumor suppressor genes and oncogenes have been well published, the exact promoter region and mechanism involved in the change of methylation pattern still remain unclear for genes such as RASSF1A. Current studies investigated the CpG islands on a broader region, and found there were different methylation patterns on different CpG islands even in the same gene. Also since detecting aberrant methylation could be a diagnostic indicator of cancer or a molecular marker for the early detection of cancer, it is important to know the precise promoter region of each gene.

Promoter hypermethylation has been found in DAPK-1 and BRCA2 in current studies. And an interesting methylation pattern after the transcription codon of RASS1A has also been detected. No hypomethylation was detected in any gene in current studies. This information will add to the literature regarding the relationship between aberrant methylation patterns and early cancer prognosis, and may have potential clinical benefits in cancer diagnosis, prevention and treatment.

Gene expression analysis needs to be performed in order to detect if there is any

correlation between the methylation pattern found in current studies and gene expression which can further support the involvement of epigenetic events in the transcription process. RT-PCR, western blot analysis and chromatin immunoprecipitation (CHIP) could be used to detect any change of gene expression, and the sequence associated with the transcription factors involved in such epigenetic events.

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APPENDIX A

PHENOL AND CHLOROFORM EXTRACTION PROCEDURE

Preparation of Genomic DNA from Mammalian Tissue

1. Excise an immediately mince tissue quickly and freeze in liquid nitrogen.
2. Grind 200 mg to 1g tissues with prechilled mortar and pestle, or crush with hammer to fine powder.
3. Add 500 µl grinding buffer to the tissue in a 1.5 ml Eppendorf tube and vortex for several minutes.
4. Spin the samples in a microfuge for 15 minutes.
5. Decant the solution and add 500 µl of lysis buffer and add 10 µl (6 Units) of proteinase K to each sample. Digest at 37° for 12 hours.
6. Add 500 µl of phenol to each tube. Vortex briefly and centrifuge for 10 minutes.
7. Making certain not to disturb the interphase, transfer aqueous top solution to a clean labeled tube and add 500 µl of chloroform. Vortex briefly and centrifuge for 10 minutes.
8. If a white precipitate is present at the aqueous/organic interface, reextract the organic phase and pool aqueous phases.
9. Carefully remove the top aqueous phase containing the DNA using a 200 ulpipettor and transfer to a new tube.

APPENDIX B

ETHANOL PRECIPITATION OF DNA

1. Add 1/10 volume of 3M sodium acetate, pH 5.2, to the solution of DNA. Mix by vortexing briefly or by flicking the tube several times with your finger.
2. Add 2 to 2.5 volumes of ice cold 100% ethanol. Mix by vortexing and place in crushed dry ice for 5 minutes or longer. Alternately, the tubes can be placed at -80° for 1 hour or at -20° overnight.
3. Spin 5 minutes in a microcentrifuge at high speed and remove the supernatant.
4. Add 1 ml of room temperature 70% ethanol. Invert the tube several times and microcentrifuge as in step 6.
5. Remove the supernatant. Dry the pellet in a desiccator under vacuum or in a hood.
6. Dissolve the dry pellet in an appropriate volume of water or TE buffer (10mM Tris-HCL, 1mM EDTA), pH8.0

APPENDIX C

BISULFITE TREATMENT OF DNA

This protocol was adapted from Frommer et.al.1992.

1. Dilute DNA (up to 2 mg) into 50 ml with distilled H_2O.
2. Add 5.5 ml of 2M NaOH.
3. Incubate at 37°C for 10 minutes (to create single stranded DNA).
4. Add 30 ml of 10 mM hydroquinone (Sigma) to each tube, freshly prepared by adding 55 mg of hydroquinone to 50 ml of water.
5. Add 520 ml freshly prepared 3M Sodium bisulfite (Sigma S-8890), prepared by adding 1.88 gm of sodium bisulfite per 5 ml of H_2O, and adjusting pH to 5.0 with NaOH.
6. Assure that reagents are mixed with DNA.
7. Layer with mineral oil.
8. Incubate at 50°C for 16 hours (avoid incubations of much longer duration as methylated C will start converting to T).
9. Remove oil.
10. Add 1 ml of DNA wizard cleanup (Promega A7280) to each tube and add mixture to miniprep column in kit.
11. Apply vacuum (manifold makes this convenient).
12. Wash with 2 ml of 80% isopropanol.
13. Place column in clean, labeled 1.5 ml tube.
14. Add 50 ml of heated water (60-70°C).
15. Spin tube/column in microfuge for 1 minute.
16. Add 5.5 ml of 3 M NaOH to each tube, and incubate at room temperature for 5 minutes.
17. Add 1 ml glycogen as carrier (we use Boehringer glycogen, undiluted).
18. Add 33 ml of 10 M NH_4Ac, and 3 volumes of ethanol.
19. Precipitate DNA as normal (overnight at -20°C, spin 30 mins), wash with 70% ethanol, dry pellet and resuspend in 20 ml water.
20. Treat DNA like RNA (keep cold, minimize freeze/thaws, store at -20°C)

VITA

Zhengyu Zhang was born in China. She recieved her bachelor of Art and Science in marine biology, then she switched to study genetics in cancer development which is what she is really interested. She also received a master's degree in experimental statistics in 2003 and will receive her second master's degree in biology at 2006. She wants to do researches involving cancer studies.